molecules as possible serotonin binding sites. J. Neurochem. 26:1193.
81. Bangham, A. D. 1968. Membrane models with phospholipids. Prog. Biophys. Mol. Biol. 18:29.
82. Bangham, A. D. 1970. The liposome as a membrane model. In *Permeability and Function of Biological Membranes,* L. Bolis, A. Katchalsky, R. D. Keynes, W. R. Lowenstein, and B. A. Pethica (Eds.). North-Holland, Amsterdam, pp. 195-206.
83. Bangham, A. D. 1972. Lipid bilayers and biomembranes. Annu. Rev. Biochem. 41:735.
84. Bangham, A. D. 1972. Model membranes. Chem. Phys. Lipids 8:386.
85. Bangham, A. D., M. W. Hill, and N. G. A. Miller. 1974. Preparation and use of liposomes as models of biological membranes. Methods Membr. Biol. 1:1.
86. Bangham, A. D. 1980. Development of the liposome concept. In *Liposomes in Biological Systems,* G. Gregoriadis and A. C. Allison (Eds.). Wiley, Chichester, England, pp. 1-24.
87. Thompson, T. E. and F. A. Henn. 1970. Experimental phospholipid membranes. In *Membranes of Mitochondria and Chloroplasts,* E. Racker (Ed.). Van Nostrand Reinhold, New York, pp. 1-52.
88. Tyrrell, D. A., T. D. Heath, C. M. Colley, and B. E. Ryman. 1976. New aspects of liposomes. Biochim. Biophys. Acta 467:259.
89. Kimbelberg, H. K. 1977. The influence of membrane fluidity on the activity of membrane bound enzymes. In *Dynamic Aspects of Cell Surface Organizations: Cell Surface Review,* Vol. 4, G. Poste and G. L. Nicolson (Eds.). Elsevier/North-Holland Biomedical Press, Amsterdam, pp. 205-293.
90. Pagano, R. E. and J. N. Weinstein. 1978. Interactions of liposomes with mammalian cells. Annu. Rev. Biophys. Bioeng. 1:435.
91. Ryman, B. E. and D. A. Tyrrell. 1980. Liposomes—bags of potential. Essays Biochem. 16:49.
92. Szoka, F. and D. Papahadjopoulos. 1980. Comparative properties and methods of preparation of lipid vesicles (liposomes). Annu. Rev. Biophys. Bioeng. 9:467.
93. Rahman, Y.-E. 1979. Potential of the liposomal approach to metal chelation therapy. In *Lysosomes in Biology and Pathology,* J. T. Dingle, P. J. Jaques, and I. H. Shaw (Eds.). North-Holland, Amsterdam, pp. 625-647.
94. Papahadjopoulos, D. (Ed.). 1978. *Liposomes and Their Uses in Biology and Medicine.* Ann. N.Y. Acad. Sci., Vol. 308. New York Academy of Sciences, New York.
95. Gregoriadis, G. and A. C. Allison (Ed.). 1980. *Liposomes in Biological Systems.* Wiley, Chichester, England.
96. Papahadjopoulos, D. and N. Miller. 1967. Phospholipid model membranes. I. Structural characteristics of hydrated liquid crystals. Biochim. Biophys. Acta 135:624.
97. Thudichum, J. L. W. 1884. *The Chemical Constitution of the Brain.* Baillière, Tindall, and Cox, London, pp. xii, 1, 10-21.

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Liposome Preparation: Methods and Mechanisms

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INTRODUCTION

Liposome preparations are now established as a useful model membrane system. Their demonstrated potential for delivering materials to the intracellular compartment has attracted investigators in experimental cell biology and in medicine as well. As a result, increasing interest has focused on preparation methods which permit liposome properties to be tailored for specific applications. The purpose of this chapter is to introduce the reader to a variety of methods [see also Szoka and Papahadjopoulos (1,2)].

An ideal preparation method would allow the investigator to produce liposomes from a variety of lipid components over a broad range of lipid concentrations. The vesicle size would be under experimental control, and size distribution would be relatively homogeneous. Finally, the method would require minimal time input and would not damage or contaminate the lipid.

No single preparation method encompasses all of these objectives, but there are several general methods now available, one of which will often provide a satisfactory approximation of a desired property. In this chapter we first discuss

the terminology that has evolved to describe liposome preparations and outline some characteristic and useful parameters. We then review the protocols of the more commonly used methods, compare certain properties of the resulting liposomes, and provide examples of recent applications.

TERMINOLOGY

Some specialized terminology has come into general use to describe specific parameters of liposome preparations and it is worthwhile to define such terms here since they will often be used later in the chapter. The first set of terms describes properties of individual vesicles and the second describes properties of liposome suspensions.

A *liposome* is defined as any structure composed of lipid bilayers that enclose a volume. The lipid is not necessarily phospholipid but this is the most commonly used component. Liposomes are characterized by their lipid composition. In mixed lipid vesicles, composition has been described in terms of mole fraction, mole ratio, or the mass ratio of one lipid to another. However, expressing the vesicle lipid composition in terms of stoichiometric proportions is more useful than mass ratios when comparing the lipid composition. Furthermore, lipid composition is more conveniently expressed as the mole fraction of total lipid rather than as the mole ratio. For example, assume that one is comparing some property of liposomes as a function of lipid composition in which a liposome preparation (3 mM total lipid) composed of 1.5 mM phosphatidic acid and 1.5 mM phosphatidylcholine is contrasted with one of 2 mM phosphatidic acid and 1 mM phosphatidylcholine. The mole ratios of phosphatidic acid:phosphatidylcholine are 1:1 and 2:1, respectively, and the mole fractions are 0.5:0.5 and 0.67:0.33. Mole fractions are more descriptive of the difference in individual bilayer composition and the total amount of each lipid in suspension. The contrasts between liposome preparations of different composition are also conceptualized more readily. This becomes particularly apparent if one is dealing with lipid mixtures of three or more components.

When phospholipids are dispersed in an aqueous phase, a heterogeneous mixture of vesicular structures is usually formed, most of which contain multiple lipid bilayers forming concentric spherical shells. These were the liposomes first prepared by Bangham and co-workers (3) and now called *multilamellar vesicles* (MLVs). If a lipid dispersion is sonicated, the MLVs are reduced to much smaller structures in the size range 25-50 nm diameter. These are termed *small unilamellar vesicles* (SUVs) since they contain only a single bilayer. Both MLVs and SUVs have certain limitations as model systems and more recently several laboratories have been able to produce vesicles in the size range 100-500 nm diameter. These are called *large unilamellar vesicles* (LUVs).

There are two important parameters of liposome preparations that are func-

tions of vesicle size and concentration. The first we will term *captured volume,* defined as the volume enclosed by a given amount of lipid and with units of liters entrapped per mole of total lipid (1 mol^{-1}). The captured volume naturally depends on the radius of the liposomes produced by a given technique and it is important to be aware that the vesicle radius and hence the captured volume are affected by the lipid composition of each vesicle and the ionic composition of the medium. Phosphatidic acid vesicles, for instance, are typically smaller than phosphatidic acid:phosphatidylcholine mixes. These effects are relatively small and for any given lipid composition the captured volume is independent of lipid concentration over a wide range. The second parameter is *encapsulation efficiency* defined as the fraction of the aqueous compartment sequestered by bilayers. The encapsulation efficiency is directly proportional to the lipid concentration; when more lipid is present, more solute can be sequestered within liposomes. The latter two parameters are important to liposome preparation methods for several reasons. For example, if liposomes are used for intracellular delivery of some entrapped compound, an LUV preparation with high captured volume will deliver a much greater volume per lipid mass than a SUV preparation. Furthermore, if the substance is costly, it is clearly desirable to maximize the amount trapped in the liposome volume.

Calculated values for captured volume and encapsulation efficiency are given in Figures 1 and 2. Note that a typical LUV preparation has a captured volume approximately 10 times that of SUVs, and that a 60 mM LUV preparation will encapsulate approximately half of the original volume. (It is interesting to note that the maximum captured volume possible for a liposome preparation is in a single large vesicle of 1 mol of lipid that is 260 m in diameter and encapsulating 6 billion liters.)

There are several methods for determining these parameters, either by direct measurement of an encapsulated compound or electron microscopic observation of individual vesicles. These are discussed later in the section "Ancillary Methods."

ARTIFACTS

Several kinds of artifacts can be introduced into liposome preparations by the methods used to produce and store them. The most common artifacts result from contamination by organic solvents and detergents, lipid peroxidation, and hydrolysis products (fatty acids and lysophosphatides), and trace amounts of polyvalent metals. These have not been investigated in detail but some accumulated practical knowledge can be summarized here.

Solvent Artifacts

Solvent contamination in liposomes can affect experimental results and it is usually worthwhile to reduce trace organic solvent concentrations to minimal levels.

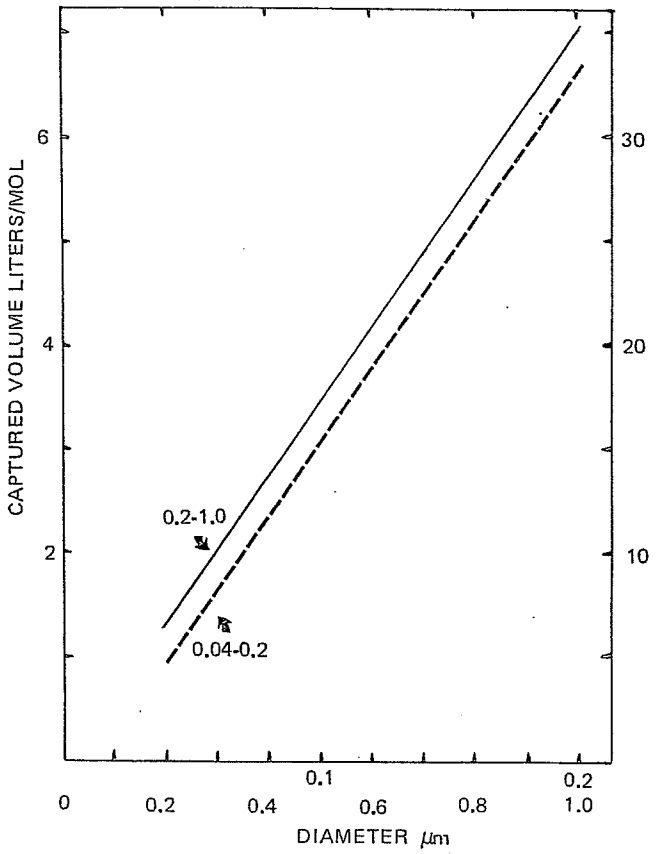


Figure 1 Captured volume of theoretical ideal unilamellar liposome preparations. The lower dashed line shows calculated captured volumes of homogeneous liposomes ranging from 0.04 to 0.2 μm in diameter (left ordinate), and the upper solid line for liposomes between 0.2 and 1.0 μm in diameter (right ordinate).

Trace chloroform is best reduced by an overnight vacuum evaporation (<10 mbar) of the dried lipid before it is used for liposome preparation. It is important to flush the evacuating apparatus with some inert gas such as nitrogen or argon to remove residual molecular oxygen that might readily peroxidize the dried lipids.

The concentrations of other solvents such as ethers and alcohols are reduced by gel filtration or dialysis. However, there are few data on how much can be removed and some investigators have been surprised by the amount of labeled ethanol that remains with liposomes following filtration. In one study (4) diethyl

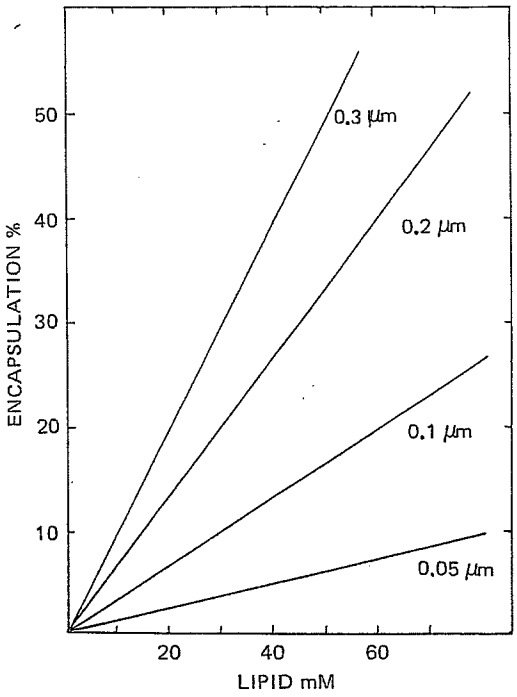


Figure 2 Calculated encapsulation efficiencies for varying concentrations of idealized liposomes. Four homogeneous preparations are shown, with diameters of 0.05, 0.1, 0.2, and 0.3 μm.

ether was shown to be reduced by gel filtration from saturating concentrations (about 0.5 M) to undetectable levels, but the error was such that concentrations up to 1 mM would not have been detected.

In practice, one cannot be certain that all traces of a solvent have been removed. However, it is possible to add small amounts of the potential contaminent to determine whether it affects the parameter being measured. If there is no effect, one may tentatively conclude that traces of that contaminant probably will not produce artifactual data.

Detergents

Cholate and deoxycholate have been used in several preparation methods (5,6). These detergents are typically removed by dilution, dialysis, BioBead adsorption, or gel filtration and have been shown to be reduced to as little as 0.1 mol%. Triton X-100 has also been used in liposome and membrane reconstitution methods

and can be removed by BioBead filtration (7,8). A 10 mM concentration can be reduced to about 0.1 mM in a single passage through the column.

More recently octyl glucoside has been found to produce liposomes in a useful size range (9). This detergent has the advantage that it is readily reduced to as little as 0.25 mol% after dialysis and gel filtration.

Divalent and Polyvalent Cations

Surprising amounts of trace metal ions can be introduced into a liposome preparation in which buffers and salts are used. Suppose that liposomes are prepared in 0.1 M NaCl. Reagent-grade NaCl contains about 0.005% of calcium, magnesium, iron, and lead, but the final concentrations of these ions in solution will be a few micromolar, significant in some investigations. Some workers include ethylenediaminetetracetic acid (EDTA) in experimental solutions to reduce the possible effects of trace cations.

Peroxidation Damage and Hydrolysis Products

Unsaturated lipids can undergo peroxidation while stored in solution or even during their isolation and purification. There will certainly be a small amount of peroxidation damage in any unsaturated lipid mixture and at best one may reduce the peroxidation to levels that do not affect the study. This can be achieved by using freshly isolated lipid or synthetic lipids that are not labile to peroxidation. Storage should be in chloroform under nitrogen at -20°C and liposome preparation should be done in an inert atmosphere. Diethyl ether readily accumulates peroxides and if it is used in a preparation method it should be freshly distilled over sodium bisulfite or otherwise purified. A simple test for lipid peroxidation is to monitor the ultraviolet (UV) absorbance ratio at 215 nm/230 nm, which measures diene conjugation produced by oxidative damage (10).

A second potential problem in liposome preparation is hydrolysis. Again, small quantities of hydrolysis products are inevitably present in typical lipid mixtures and one must therefore attempt to minimize possible artifacts. If it can be shown that trace amounts of fatty acids or lysophosphatides affect the liposome property that one is studying, the lipids should be purified by chromatography just before use.

At least one class of lipids does not undergo oxidation or hydrolysis. Branched-chain dialkyl ethers are found in primitive photosynthetic cells such as the halobacteria (11), and should be explored for their potential in liposome preparations. Such lipids serve as a useful control to determine the extent to which peroxidation and hydrolysis contribute to liposome properties.

METHODS

The major methods to be discussed are those that produce MLV, SUV, and LUV preparations. We will also discuss several other preparations that include liposomes composed of single-chain amphiphiles and model phospholipids. Each technique will be considered in terms of the number of lamellae the liposomes have, sources of contamination, ease of preparation, encapsulation efficiency, and captured volume.

Small Unilamellar Vesicles

The first SUV lipid dispersions were prepared by sonication about the same time that MLVs were introduced (12,13). They have since been developed into a standard system for investigating biophysical aspects of lipid bilayers (14-16). In a typical preparation, phospholipid in chloroform is dried under nitrogen and rotary-evaporated to remove trace solvent. The desired aqueous phase is added to produce a lipid concentration in the millimolar range, after which the suspension is sonicated to clarity at 45°C in a probe or bath sonication unit. This might require only a few minutes with the probe but as much as 2 hr with bath sonication. Although it takes considerably longer, the bath sonication has the advantage that it can be carried out in a closed container under nitrogen or argon, does not contaminate the lipid with metal from a probe tip, or produce significant heating. When using a bath sonicator it is important that the closed container be located precisely in the nexus of the ultrasonic waves. At this focal point of input power the lipid dispersion inside will be vigorously and visibly agitated.

When the resulting dispersion is examined by negative staining electron microscopy it is found to contain vesicles ranging from 25 to 50 nm in diameter. Huang (17) showed that these can be brought to near homogeneity by gel filtration. When this is performed, a highly uniform preparation of vesicles in the size range 25 nm is produced. This remains the most homogeneous liposome preparation. A summary of captured volume and encapsulation efficiencies is given in Table 1.

A second method for producing SUVs was developed by Batzri and Korn (18). In this procedure an ethanol solution of lipid is injected quickly into the desired aqueous phase to a final lipid concentration of 3-30 mM. The resulting vesicles range from 30 to 110 nm in diameter, depending on the lipid concentration. A potential drawback of this method is that high concentrations of ethanol must be removed by a subsequent purification step.

A third method for producing SUVs is to pass MLVs through a French press at 20,000 psi four times (19). Once again, the resulting product is in the range 30-50 nm in diameter. This preparation method has not been extensively studied but is well worth pursuing since it sidesteps some of the problems associated with other SUV methods.

Table 1 Summary of Characteristics of Typical Liposome Preparations

Lipid composition[a]	Diameter (μm)	Captured volume (liters/mol)	Encapsulation efficiency (% original volume)	Reference
Small unilamellar vesicles				
PC:PA 90:10 (1 mM)	–	0.8	–	23
PC:DCP:Chol 70:20:10	–	0.7	–	24
PS 20 mM	0.03	0.5	1	32
PC 20 mM	0.03	0.23	–	6
Large unilamellar vesicles				
PC:PA 90:10 (1 mM, diethyl ether injection)	0.05-0.25	17 ± 4	1.7	23
PC:DCP:Chol 7:2:1 (15 mM, ether injection)	0.1-0.4	23-31	38-46	24
PG:PC:Chol 1:4:5 (66 mM, REV method)	0.2-1.0	11.7	35-65	25
PS 20 mM (PS-cochleate method)	0.2-1.0	7	15	32

PC 50 mM (DOC + dialysis)	0.1	2.4	12	6
PC 16 mM (octyl glucoside + dialysis)	0.2–0.3	7–7.4	22	9
Soybean phospholipid 20 mg/ml (FTS method)	–	8	–	34
Single-chain amphiphile (dodecyl sulfate-dodecanol 50 mM)	1–10	–	42	36
Multilamellar vesicles				
PG:PC:Chol 1:4:5 (66mM)	0.4–3.5	3.5	5–15	25
PC:DCP:Chol 7:2:1 (15 mM)	–	6.9	10.4	24
PS 20 mM	–	2.5	5	32
PC:PA 90:10 (1 mM)	–	1.8	–	23

[a]PC, phosphatidylcholine; PA, phosphatidic acid; DCP, dicetyl phosphate; PS, phosphatidylserine; PG, phosphatidylglycerol; DOC, deoxycholate; Chol, cholesterol.

Fendler (10) has shown that dialkyl surfactant compounds, dioctadecyldimethylammonium chloride (DODAC) and dihexyldecylphosphate (DHP), are capable of forming vesicular structures and has termed the resulting vesicles "membrane mimetic agents." In a typical preparation the vesicles are formed by simple sonication. They have most of the properties of SUV liposomes produced from naturally occurring lipids and offer a useful alternative model system. In particular, the DODAC vesicles carry a positive charge which is not available when natural lipids are used.

The homogeneity of SUV preparations makes them a preferred system for biophysical studies of lipid bilayers. Because of their small diameter and low captured volume they are of limited value as mediators of intracellular solute delivery. Also, due to their high radius of curvature, SUVs may have special properties not shared by more planar membranes, and in some applications this could limit their usefulness as a model membrane system.

Multilamellar Vesicles

Bangham and co-workers (3) first recognized that lipid vesicles were models of cell membranes, particularly with regard to permeability studies. Their liposome system, now described as multilamellar vesicles (MLVs), is still used in certain applications. In a typical preparation, 10 mg of egg phosphatidylcholine containing 5 mol% of egg phosphatidic acid is evaporated from chloroform to produce a thin film on the wall of a 100-ml round-bottom flask. (The phosphatidic acid adds a negative charge to the lipid which aids hydration and inhibits aggregation.) The flask is placed under vacuum overnight to remove any remaining chloroform. The desired aqueous solution is added (5 ml) and the lipid is hydrated for several hours. The milky suspension is then swirled or vortexed to disperse the lipid.

In a variation of this method, Reeves and Dowben (21) hydrated the dried lipid with wet nitrogen gas that had passed through a water phase, followed by addition of water and gentle swirling. The slower hydration was found to produce very large vesicles up to several hundred micrometers in diameter, but only in the absence of ions or protein.

(The process of lipid hydration is an interesting classroom exercise and an esthetic pleasure. Lipid hydration can be observed under a phase or polarizing microscope. A drop of lipid in chloroform is dried on a slide, a coverslip is placed over it, and a drop of buffer solution is added from the side. Over a period of 30-60 min, a beautiful array of myelin figures grows out of the lipid during hydration, often forming remarkably complex geometric structures.)

Dehydration Formation of Multilamellar Vesicles

The following MLV preparation method is unpublished and utilizes dehydration to cause vesicle fusion. The resulting liposomes are oligolamellar

and multilamellar and can efficiently encapsulate large macromolecular structures.

One to ten micromoles of phospholipid is dispersed by sonication in 1 ml of water. The material to be encapsulated is added and the water is evaporated under a dry nitrogen stream. As the liposomes are concentrated by the drying process, they fuse to form multilamellar planes and thereby sandwich the added material between the resulting lamellae. After drying is complete, the lipid is rehydrated by placing a moist cotton plug in the tube or by flushing the lipid with a stream of hydrated nitrogen for a few hours. Water is then added and the mixture is vortexed to disperse the lipid vesicles. The resulting liposomes can be sized by passing them through polycarbonate filters. This method has the advantage that it can be used with most phospholipids and does not require any special apparatus other than a sonicator. The vesicles have an encapsulation efficiency of nearly 50% for DNA (1 mg of DNA to 10 mg of lipid) but are not well suited for trapping smaller molecules.

The major advantage of MLV preparation is the simplicity of the procedure and the fact that it is applicable to a wide variety of different lipid mixtures. Certain studies may even require multilamellar structure. For instance, it is likely that the particulate structures seen in some liposomes by freeze-fracture represent points at which neighboring lamellae touch; hence, studies examining this phenomenon require MLVs (22).

The captured volume of MLVs is variable due to changes in lipid composition and hydration techniques. As seen in Table 1, values range from 1.8 (23) to 6.9 liters/mol (24). However, the multilamellar character and heterogeneity of MLVs is a distinct disadvantage in many applications. The MLV character is particularly confusing when working with permeability studies since it is not certain how many of the lamellae are involved in permeation of the fluxing species. Problems may also arise in liposome fusion studies with cells since large amounts of lipid are delivered along with the liposomal content.

Large Unilamellar Vesicles

It became apparent in the 1970s that larger liposomes than SUVs would be useful and therefore several investigators began to explore various methods for preparing LUVs. The three most commonly used principles are infusion procedures, reverse-phase evaporation, and detergent dilution. In the infusion method, a lipid solution in a nonpolar solvent is infused into an aqueous solution under conditions that cause the solvent to caporize, resulting in the formation of liposomes. In the reverse-phase evaporation (REV) method, a dispersion of inverted micelles of lipid is produced in a system containing a mixed organic and aqueous phase. As the solvent is evaporated away, the micelles coalesce to form liposomes. Finally, a variety of methods use different means to dilute detergent originally codispersed with the lipid. This principle has been applied to the first

commercially available liposome preparation apparatus. These three general methodologies are described in the following section.

Infusion Methods

An infusion method utilizing diethyl ether was published by Deamer and Bangham (23) and has been developed further over the past few years (24). This method depends on the fact that a solution of lipid in a nonpolar solvent can be infused into an aqueous phase under conditions that cause the solvent to vaporize. The lipid is left as a thin film at the interface of the vapor bubble and the aqueous phase. It can be calculated that the film is composed of only a few bilayers of lipid. As the bubble rises through the aqueous phase, the lamellae are dispersed to form uni- and oligolamellar liposomes. These are then sized by passage through polycarbonate filters.

In a typical run, 4 ml of lipid solution in pentane or diethyl ether (1-2 mM lipid) is infused slowly (0.2 ml/min) into 4 ml of aqueous phase warmed to 60°C. This phase is contained in a converted Leibig condenser that has warm water circulating through the outer jacket. The aqueous phase is flushed with a gentle stream of nitrogen bubbles, which promotes solution mixing, provides an inert atmosphere at the air-solution interface, and increases the size of the vapor bubble, thereby favoring formation of unilamellar vesicles. When infusion is complete, the liposomes are filtered through a polycarbonate filter to size them and remove aggregates. This step is important to most of the LUV methods and it is likely that larger oligolamellar vesicles are sized down as they are extruded through the pores of the filter. Following this step, the liposomes are put through a gel filtration step (i.e., Sephadex G-25 or G-50, Biogel P-10) to remove any remaining solvent and to exchange the unencapsulated aqueous phase if desired.

The advantage of the infusion procedure is that it is applicable to a wide variety of lipids and can be completed in less than an hour. The main limitation is that the resulting preparations are relatively dilute (1-15 mM lipid) so that the encapsulation efficiency is low. (However, Schieren et al. (24) reported that they could obtain 35% encapsulation efficiency using pet ether infusion and 30 mM final concentration.) If the temperature of infusion might damage heat-sensitive materials, it is possible to place the column under vacuum so that lower temperatures can be used.

Reverse-Phase Evaporation

Szoka and Papahadjopoulos (25) have designed an alternative LUV methodology in which lipid in mixed aqueous-nonpolar solvent forms inverted micelles, such that the lipid tails are inserted into the nonpolar phase and the head groups surround water droplets. As the nonpolar solvent is evaporated away under vacuum the micelles coalesce to form large uni- and ologolamellar vesicles. In a typical preparation, 66 μmol (50 mg) of lipid is dispersed by bath sonication in a 1 ml:3

ml mixture of aqueous solution-diethyl ether. The ether is then removed by rotary evaporation under vacuum followed by polycarbonate filter sizing and gel filtration to remove trace solvent.

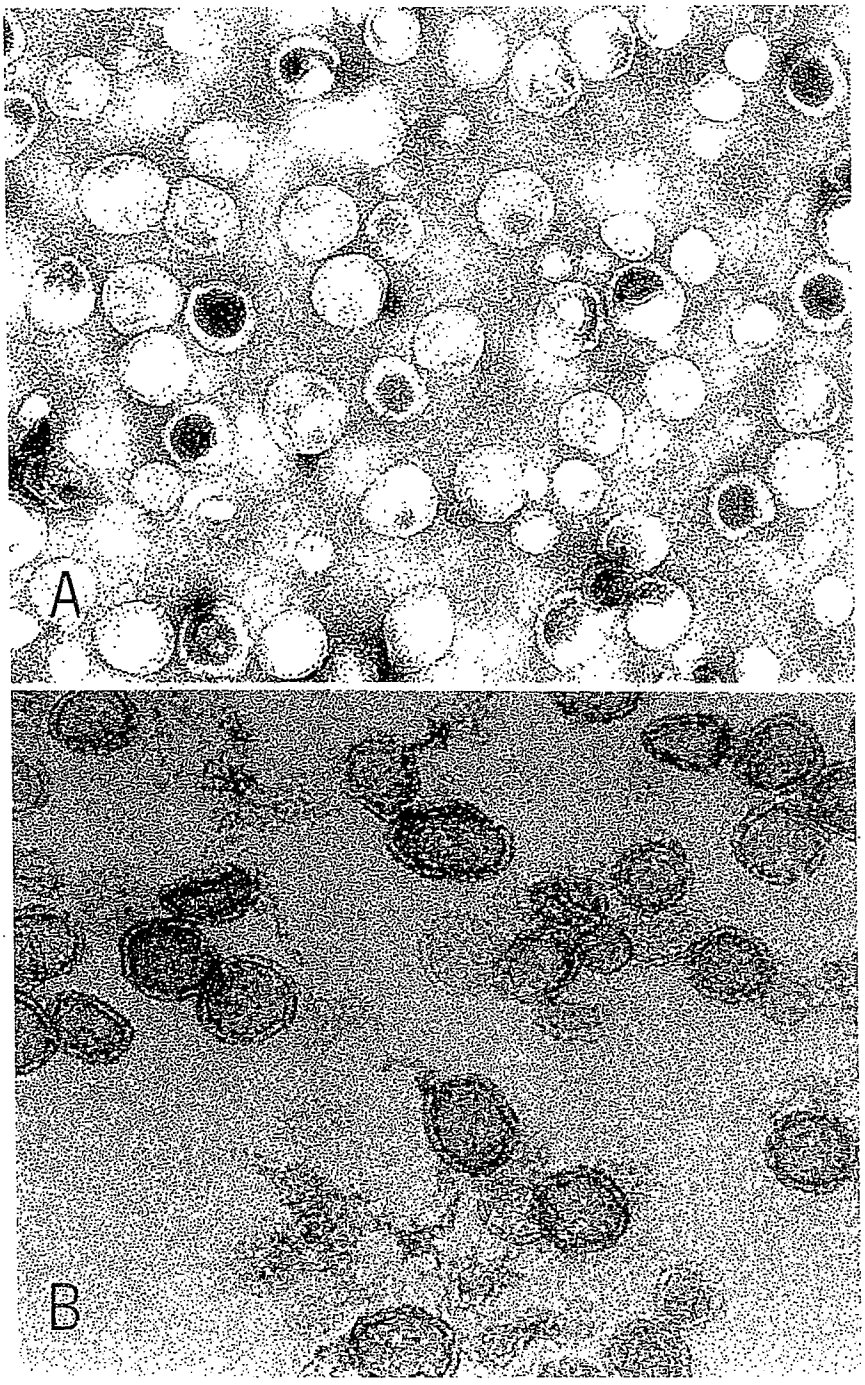
The REV method has the distinct advantages of being applicable to a range of volumes (as little as 0.2 ml) and in its ability to encapsulate a large fraction of the original volume (up to 65% in low-ionic-strength media). Its disadvantages are that components are exposed to organic solvents and sonication which may be damaging and that the lipid requirement (10 mg per 0.2 ml of solution) is relatively large.

Detergent Dilution

Detergents such as the bile salts (cholate and deoxycholate) and Triton X-100 have been applied to the solubilization and reconstitution of biological membranes with considerable success. In general, the detergent is added until the membrane suspension, due to the formation of mixed micelles of detergent, lipid, and protein, has clarified. The detergent concentration is then lowered and the original lipid and protein form vesicular membrane structures, often with reconstituted function of the protein.

Detergents have been used less often as a primary liposome preparation method. However, these procedures do have certain advantages. First, they are relatively gentle in their action and would not be expected to hydrolyze or peroxidize liposome components, and second, the detergent/lipid ratio is readily varied, which permits considerable experimental control over the size of the resulting vesicles. In a typical detergent-LUV method (6), egg phosphatidylcholine is mixed with deoxycholate in 1 ml of aqueous solution (20 μmol of lipid per 10 μmol of detergent). After a few minutes of sonication in a bath sonicator, the originally turbid suspension becomes opalescent due to the presence of mixed micelles. The deoxycholate is then removed by gel filtration. These vesicles are about 100 nm in diameter and would be considered small LUVs. They have a captured volume of 2-3 liters/mol and successfully encapsulate cytochrome *c* and glucose. They do not trap radiolabeled sodium chloride unless it is present during the removal of deoxycholate.

Octyl glucoside has been employed by Mimms et al. (9). In this procedure, egg phosphatidylcholine is dried as a film in a glass container and 0.4-0.6 ml of octyl glucoside solution is added. In typical experiments, the amount of lipid can be varied from 2 to 10 mg, and the optimal detergent/lipid ratio is in the range 10:1 to 15:1. The solution is briefly sonicated and the clear mixture is dialyzed twice for 12 hr against 1 liter of buffer which includes the compound to be encapsulated. During dialysis, the solution becomes turbid and examination shows that vesicles ranging around 0.2 μm in diameter are formed. The captured volume ranges from 6 to 7.5 liters/mol and cytochrome *c* trapping demonstrates a 22% encapsulation efficiency. After two gel filtrations, the detergent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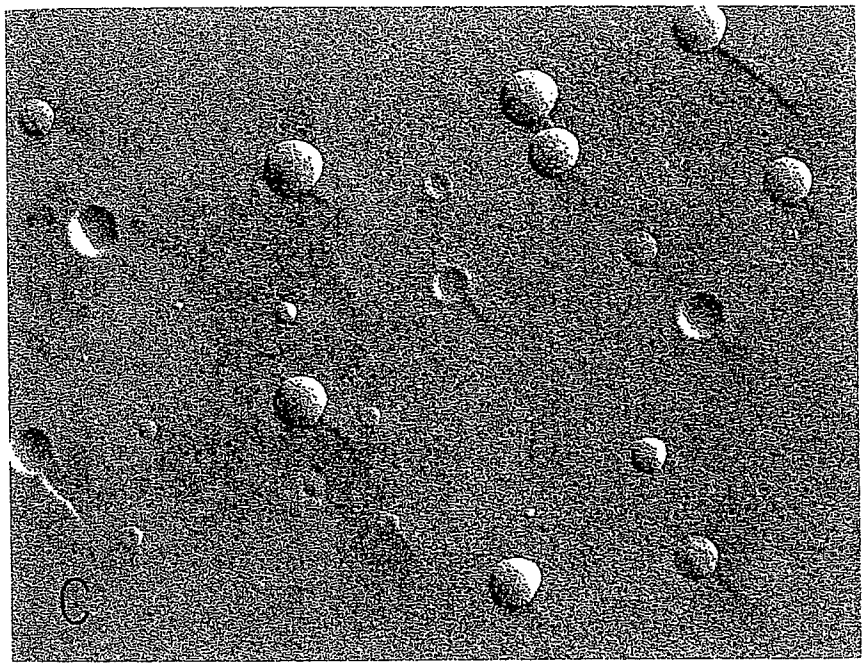


Figure 3 Electron micrographs of LUV preparations: (A) Negative staining; (B) Thin sections; (C) Freeze-fracture. Prepared by the Lipoprep apparatus. (From Ref. 27.)

is reduced to about 1 octyl glucoside molecule per 400 phospholipids molecules. The measured permeability to monovalent cations and anions is in the range observed for SUVs, suggesting that this amount of detergent does not markedly affect the permeability barrier.

Detergent removal has been applied in the only commercial liposome apparatus on the market, called Lipoprep. This device is based on the work of Milsmann et al. (26) and Zumbuehl and Weder (27). In the Lipoprep method, phospholipid is dispersed with detergent in ratios ranging from 1:1 to 2:1. The detergent is removed by passing the mixture through a rapid-flow dialysis cell. The size of the vesicles prepared with cholate is 50-80 nm in diameter (Fig. 3) and the captured volume ranges from 1.8 to 2.4 liters/mol. If octyl glucoside is used rather than

cholate, the diameter increases to 180 nm (Fig. 4). The vesicle diameter can be varied from 80 to 200 nm in a predictable and homogeneous fashion by changing the ratio of octyl to heptyl glucoside (28). The homogeneity and the ability to manipulate the mean diameter of the vesicle preparation is a significant advantage of the Lipoprep method, since a number of investigations involve studies of liposome properties that are related to vesicle diameter.

The detergent dilution methods are widely applicable in liposome preparations, particularly when proteins are to be reconstituted into the resulting membrane. The methodological limitations are that preparations using dialysis require hours or even days to be completed, and if the solute to be entrapped is dialysis membrane permeant, the encapsulation efficiency is very low.

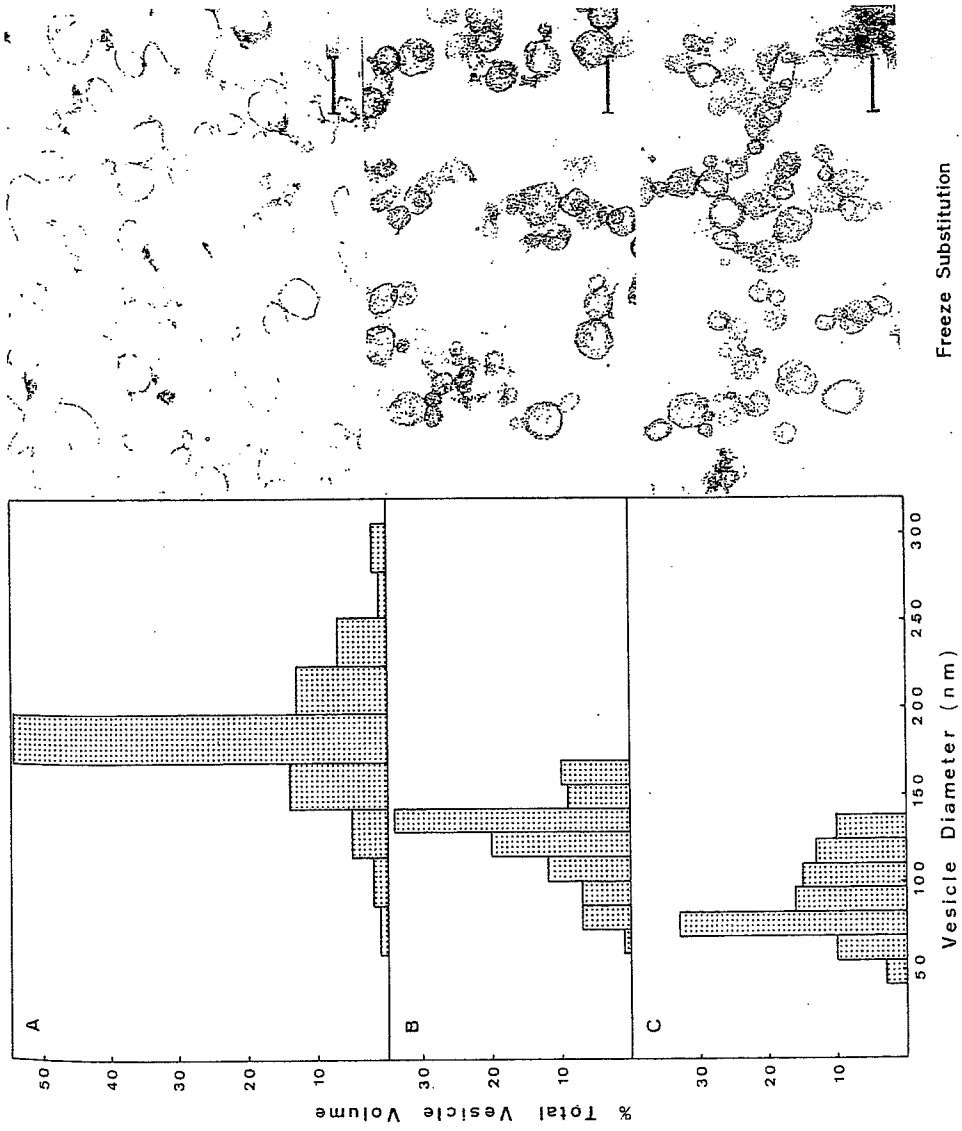
Fusion Methods

Phosphatidylserine Cochleate Vesicles If SUVs can be caused to fuse, the fusion process should be able to encapsulate some fraction of the material dissolved in the aqueous phase. Papahadjopoulos et al. (29) mixed SUVs composed of negatively charged phospholipids with calcium followed by EDTA to chelate the calcium. When calcium ion interacts with phosphatidylserine SUVs, the vesicles first aggregate to form a dense precipitate and then fuse to produce multilamellar arrays that often take the form of cochleate cylinders. When the calcium is removed by EDTA, the structures swell and form large uni- and oligolamellar vesicles.

In a typical experiment, 1-10 mM unsaturated phosphatidylserine is used to prepare SUV. Sufficient calcium chloride is added to produce a final calcium/lipid ratio of 1:2 and precipitate the SUVs. To disperse the precipitate, EDTA is added in slight excess with stirring until the precipitate forms an opalescent suspension of LUVs. The resulting vesicles are able to encapsulate structures as large as viruses (30), nucleic acids (31), and ferritin (32) under relatively mild conditions. The captured volume is in the range of 7 liters/mol, and the encapsulation efficiency is 10-15% in a 20 mM lipid dispersion.

Freeze-Thaw-Sonication A second fusion method is the freeze-thaw-sonicate (FTS) technique. This method was first explored by Kasahara and Hinkle (33) for reconstituting functional membranes and Pick (34) has shown that it is applicable to liposome preparation. In a typical procedure, 30 mg of lipid is dispersed by sonication in 1 ml of buffer, then mixed with the material that is to be encapsulated. The mixture is frozen in liquid nitrogen, thawed and bath-sonicated for 30 sec. (The sonication serves primarily to disperse aggregated material.) The resulting vesicles are reasonably impermeable to ions and have captured volumes in the range of 8 liters/mol. The FTS method has the advantage of being a relatively

Figure 4 Diameters of liposome preparations. Bar shows 0.2 μm. (From Ref. 28.)


Freeze Substitution
A
B
C
50
40
30
20
10
30
20
10
30
20
10
% Total Vesicle Volume
50
100
150
200
250
300
Vesicle Diameter (nm)

simple and gentle procedure. However, it does not work well in the presence of sugars, high concentrations of ions, or divalent cations.

Single-Chain Amphiphiles

It has recently been found that many single-chain amphiphiles form vesicles under certain conditions. This was first demonstrated by Gebicki and Hicks (35) for unsaturated fatty acids, and later extended by Hargreaves and Deamer (36) to include other single-chain amphiphiles.

To produce oleic acid liposomes, a 50 mM micellar solution of oleate is prepated by titrating oleic acid to pH 10 with 0.1 M NaOH. The pH is then carefully lowered to pH 8.5. At this pH the suspension becomes opalescent and if examined by phase microscopy is found to contain vesicular structures. It is likely that the stability of the vesicles depends on the formation of an acid soap at pH 8.5 and that hydrogen bonding stabilizes the structure through oleate-oleic acid complexes.

A striking demonstration of single-chain amphiphile vesicles was provided by Hargreaves and Deamer (36). These investigators showed that dodecyl sulfate is capable of forming liposomes if the equivalent of an acid soap is produced. However, instead of titrating, dodecyl alcohol is added to introduce an uncharged group capable of forming hydrogen bonds with the dodecyl sulfate. In a typical preparation, a 2:1 dodecyl alcohol:dodecyl sulfate mixture (50 mM total amphiphile in aqueous buffer) is sonicated briefly at 55°C. Examination of the mixture reveals vesicles in the size range 0.1-1.0 μm, and after a few hours, fusion events produce even larger vesicles which are remarkably stable. For instance, if carboxyfluorescein is included in the mixture, the vesicles can be separated from the external phase by gel filtration and the dye remains encapsulated over a period of several weeks. The encapsulation efficiency of a 50 mM total amphiphile dispersion is about 40%.

ANCILLARY METHODS

A number of practical methods have evolved for working with liposome preparations and monitoring their properties. These cannot be covered in any detail in a short chapter, but it seems appropriate to provide brief descriptions and references to specific methods which have proven useful in the laboratory. These can roughly be divided into methods involved in liposome preparation and methods used in analysis.

Preparation Methods

Gel Filtration

Most investigators are familiar with standard gel filtration columns that can be used to size liposomes and to exchange the external volume. One disadvantage

of such methods is that they are often slow and always produce considerable dilution of the liposomes. Fry and co-workers (37) have published a method which does not dilute the sample and is much more rapid, especially if multiple samples must be run. In this, the desired gel is prepared in a plastic syringe tube. The tube is then placed in a conveniently sized centrifuge tube and centrifuged once at a low *g* force to remove all void volume solution. The liposome preparation is placed on top of the gel, followed by a second low-speed centrifugation. The undiluted liposomes appear in the bottom of the tube, leaving the original external phase entrapped in the internal gel volume at the top. It is important to determine empirically the time and *g* force of the low-speed centrifugation for the amount and type of gel used. The retention of the void volume is very dependent on the individual gel characteristics.

Sizing Liposomes

As mentioned earlier, it is convenient to use polycarbonate filters to size a heterogeneous liposome preparation. The liposome dispersion is placed in a glass syringe and forced through the filter under gentle pressure. The filter can range from 0.1 to 1.0 μm, depending on the final size desired. Simple centrifugation can be used to separate SUVs from MLVs, and as described by Huang (17), gel filtration can then size the SUVs to near homogeneity.

Concentrating Liposomes

Occasionally, it is necessary to concentrate liposomes. Sometimes vesicles can be pelleted by high-speed centrifugation but this will not always work with SUV and LUV preparations. An improved way to carry this out is to add a small amount of protamine or polylysine until an obvious flocculant precipitate has formed (38). This can be centrifuged to form a pellet. The pellet can be dispersed by the addition of heparin, which competes with the protamine for binding sites and releases the liposomes. This method is useful only if the liposomes have at least some small amount of net negatively charged lipid (such as 5% phosphatidic acid) and if the presence of small amounts of protamine and heparin do not affect desired vesicle properties.

Monitoring Liposome Properties

Light Microscopy

Sometimes liposomes are large enough to be viewed by phase microscopy (1-10 μm in diameter). However, depending on conditions, the liposomes may not have sufficient phase contrast to be seen readily. We have found that the addition of sucrose or dextran to such dispersions produces a phase contrast so that

the vesicles appear phase bright against a dark background (36). An alternative procedure is to include a small amount (<0.5 mol%) of a fluorescent lipid analog and view the liposome preparation by fluorescence microscopy.

Electron Microscopy

Negative staining and freeze-fracture methods are most commonly applied to liposome preparations and the techniques are well known. We have found it useful in negative staining of unsaturated LUVs to prefix with OsO_4 before staining. This prevents the distortion often caused in larger vesicles by the staining process. We have also found that etching of freeze-fracture specimens can provide information about the fracture of vesicles that are unilamellar in a given preparation. In standard freeze-fracture preparations, the vesicles appear only if the fracture plane follows the membranes and cross fractures are not obvious. However, after 5 min of etching, cross-fractured vesicles are clearly seen and the number of lamellae can readily be determined (Fig. 5).

Captured Volume and Encapsulation Efficiency

For simple estimates of captured volume in high-ionic-strength media, liposomes can be prepared in 0.1 M sodium or potassium chromate. Following gel filtration to remove the external chromate, the trapped chromate can be determined by solubilizing an aliquot of the liposomes in 10 mM Triton X-100, followed by measuring A_{380} for chromate. A simple calculation then provides an estimate of captured volume as liters per mole of lipid. The ratio of chromate concentration in the aliquot (Triton added) to the original chromate concentration is divided by the actual lipid concentration of the aliquot. Thus, it is also necessary to determine the final lipid concentration in order to determine the captured volume. The same data can be used to calculate the encapsulation efficiency. The captured volume multiplied by the actual lipid concentration (X100) gives the encapsulation efficiency. For low-ionic-strength media, 6-carboxyfluorescein can be used as a marker (0.1 mM) and its fluorescence measured to determine the amount entrapped.

Liposome-Liposome Interactions

Since liposomes are dynamic structures, it is worthwhile considering some interactions between vesicles in suspension which might affect a liposome property under study. We conclude this chapter by considering briefly unimolecular exchange of lipid, liposome aggregation, and liposome-liposome fusion.

Unimolecular disassociation from one bilayer, diffusion, and reassociation

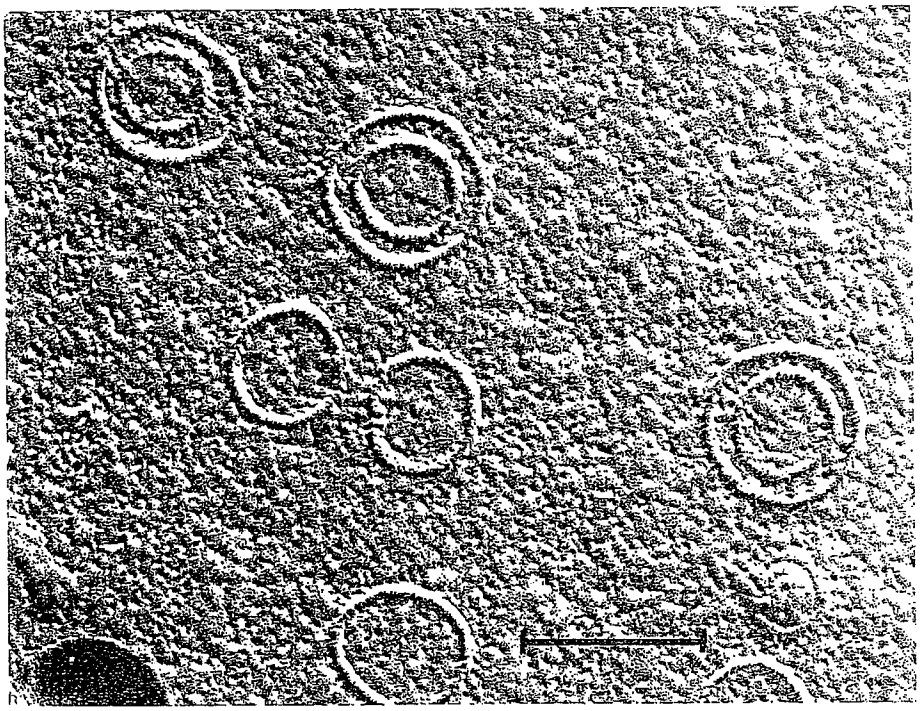


Figure 5 Freeze-etch image of cross-fractured liposomes. Phosphatidylserine liposomes were prepared by diethyl ether infusion method in 10 mM phosphate buffer, pH 7.5. Bar shows 0.2 μm.

can result in changes in vesicle composition. For instance, it has been shown that monomolecular phosphatidylcholine is in equilibrium with vesicular lipid (39,40). There is a vectorial addition of lipid from smaller to larger vesicles, or from short acyl chain vesicles to longer acyl chain pure phosphatidylcholine vesicles. It has also been shown that monomolecular lipid exchange occurs between fluorescently labeled phospholipids and the plasma membrane of cells in culture (41).

The detection of unimolecular exchange is not a trivial procedure. The investigators cited above used differential scanning calorimetry and fluorescence spectroscopy, respectively. With the advent of a variety of synthetic phospholipids that are commercially available, differential scanning calorimetry has become more practical. Other techniques using radiolabeled or fluorescent deriva-

tives of lipids have the inherent problem of separating the labeled fraction from the remainder of the lipid. If it is not possible to use changes in the monomer/excimer ratio of a labeled analog (40), our experience suggests that monitoring lipid exchange between SUVs or LUVs and MLVs is currently the strongest approach, since it is relatively easy to separate MLVs from the others by centrifugation or filtration.

Aggregation is usually prevented by imparting a net charge on the bilayer and including chelating agents in the buffer to remove trace polyvalent ion effects. For some experimental protocols it is not possible to include these safeguards and it may therefore be necessary to determine if significant liposome-liposome aggregation is occurring during the time scale of the experiment. Aggregation can be monitored by following light-scattering changes at A_{500}. Light-scattering increases also include those due to vesicle size increases from monomolecular diffusion and liposome fusion. The latter possibilities can be excluded by testing for exchange diffusion and vesicle fusion with assays described in this section. Alternatively, a fluorescence resonance energy transfer assay has been suggested which follows vesicle aggregation (42).

The third possible liposome-liposome interaction that we will consider is that of fusion. Two methods are now available which follow the mixing of the entrapped aqueous compartments or mixing of the lipid components. The first assay was developed by Wilschut and Papahadjopoulos (43) and monitors the formation of a complex between the lanthanide ion, terbium, and dipicolinate. This complex is several orders of magnitude greater in fluorescence than the intrinsic fluorescence of terbium. Terbium citrate is trapped in one set of liposomes and sodium dipicolinate is trapped in a second population. The liposomes are then mixed and the rate and extent of fusion is followed by the increase in fluorescence.

An alternative method is to monitor quantitatively the mixing of two fluorescent lipid analogs originally in separate bilayers. This recently developed assay for vesicle fusion (44) is based on the transfer of fluorescence resonance energy from donor probe to acceptor probes in the same bilayer. If a fusion event occurs between a donor-labeled liposome and acceptor-labeled liposome, the proximity of both probes in the same bilayer causes a change in the fluorescence emission spectrum. The extent of probe intermixing can be determined quantitatively by comparing the experimental liposomes against a "mock-fused" standard. Both the aqueous compartment assay and the lipid compartment assay are adaptable to a wide range of experimental conditions and both assays are relatively insensitive to aggregation-induced artifacts.

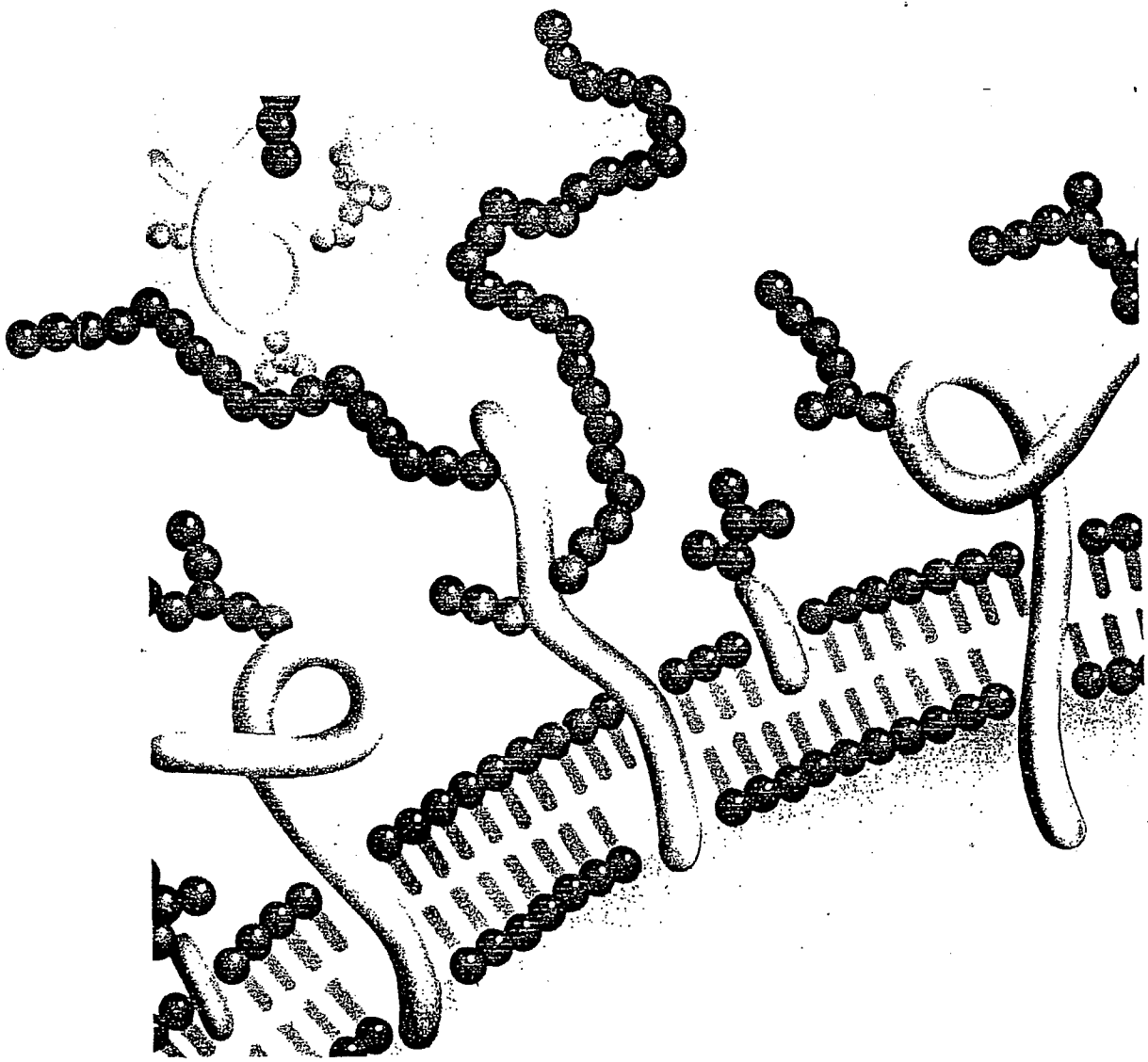
REFERENCES

1. Szoka, F. and D. Papahadjopoulos. 1980. Comparative properties and methods of preparation of lipid vesicles (liposomes). Annu. Rev. Biophys. Bioeng. 9:467.
2. Szoka, F. and D. Papahadjopoulos. 1981. Liposomes: preparation and chatacteristics. In *Liposomes: From Physical Structure to Therapeutic Applications,* G. Knight (Ed.). Elsevier/North Holland Biomedical Press, Amsterdam, pp. 51-82.
3. Bangham, A. D., M. M. Standish, and J. C. Watkins. 1965. Diffusion of univalent ions across the lamellae of swollen phospholipids. J. Mol. Biol. 13:238.
4. Deamer, D. W. 1978. Preparation and properties of ether-injection liposomes. Ann. N.Y. Acad. Sci. 308:250.
5. Kagawa, Y. and E. Racker. 1971. Partial resolution of the enzymes catalysing oxidative phosphorylation. XXV. Reconstitution of vesicles catalysing $^{32}P_i$-adenosine triphosphate exchange. J. Biol. Chem. 246:5477.
6. Enoch, H. G. and P. Strittmatter. 1979. Formation and properties of 1000-A-diameter, single-bilayer phospholipid vesicles. Proc. Natl. Acad. Sci. USA 76:145.
7. Gerritsen, W. J., A. J. Verkleij, R. F. Zwaal, and L. L. Van Deenen. 1978. Freeze-fracture appearance and disposition of band 3 protein from the human erythrocyte membrane in lipid vesicles. Eur. J. Biochem. 85:255.
8. Peterson, S. W., S. Hanna, and D. W. Deamer. 1978. Comparative studies of detergent effects on the calcium adenosine triphosphatase of sarcoplasmic reticulum. Arch. Biochem. Biophys. 191:224.
9. Mimms, L. T., G. Zampighi, Y. Nozaki, C. Tanford, and J. A. Reynolds. 1981. Phospholipid vesicle formation and transmembrane protein incorporation using octyl glucoside. Biochemistry 20:833.
10. Klein, R. A. 1970. The detection of oxidation in liposomes preparations. Biochim. Biophys. Acta 210:486.
11. Kates, M., B. Palameta, C. N. Joo, D. J. Kushner, and N. E. Gibbons. 1966. Aliphatic diether analogs of glyceride-derived lipids. IV. The occurrence of di-O-dihydrophytylglycerol ether containing lipids in an extreme halophilic bacteria. Biochemistry 5:4092.
12. Saunders, L., J. Perrin, and D. B. Gammack. 1962. Ultrasonic irradiation of phospholipid sols. J. Pharm. Pharmacol. 14:567.
13. Abramson, M. B., R. Katzmann, and H. P. Gregor. 1964. Aqueous dispersions of phosphatidylserine. J. Biol. Chem. 239:70.
14. Papahadjopoulos, D. and N. Miller. 1967. Phospholipid model membranes. I. Structural characteristics of hydrated liquid crystals. Biochim. Biophys. Acta 135:624.

15. Papahadjopoulos, D. and J. C. Watkins. 1967. Phospholipid model membranes: permeability properties of hydrated liquid crystals. Biochim. Biophys. Acta 135:539.
16. Johnson, S. M., A. D. Bangham, M. W. Hill, and E. D. Korn. 1971. Single bilayer liposomes. Biochim. Biophys. Acta 233:820.
17. Huang, C. H. 1969. Studies on phosphatidylcholine vesicles: formation and physical characteristics. Biochemistry 8:344.
18. Batzri, S. and E. D. Korn. 1973. Single bilayer liposomes prepared without sonication. Biochim. Biophys. Acta 298:1015.
19. Barenholz, Y., S. Amselem, and D. Lichtenberg. 1979. A new method for preparation of phospholipid vesicles. FEBS Lett. 99:210.
20. Fendler, J. H. 1980. Surfactant vesicles as membrane mimetic agents: characterization and utilization. Acc. Chem. Res. 13:7.
21. Reeves, J. P. and R. M. Dowben. 1969. Formation and properties of thin-walled phospholipid vesicles. J. Cell. Physiol. 73:49.
22. Miller, R. C. 1980. Do "lipidic particles" represent intermembrane attachment sites? Nature 287:166.
23. Deamer, D. W. and A. D. Bangham. 1976. Large volume liposomes by an ether vaporization method. Biochim. Biophys. Acat 443:629.
24. Schieren, H., S. Rudolph, M. Finkelstein, P. Colemen, and G. Weissman. 1978. Comparison of large unilamellar vesicles prepared by a petroleum ether vaporization method and multilamellar vesicles. Biochim. Biophys. Acta 542:137.
25. Szoka, F. and D. Papahadjopoulos. 1978. A new procedure for preparation of liposomes with large internal aqueous space and high capture by reverse phase evaporation. Proc. Natl. Acad. Sci. USA 75:4194.
26. Milsmann, M. H. W., R. A. Schwendener, and H. G. Weder. 1978. The preparation of large single bilayer liposomes by a fast and controlled dialysis. Biochim. Biophys. Acta 512:147.
27. Zumbuehl, O. and H. G. Weder. 1981. Liposomes of controllable size in the range of 40 to 180 nm by defined dialysis of lipid/detergent mixed micelles. Biochim. Biophys. Acta 640:252.
28. Schwendener, R., M. Asanger, and H. G. Weder. 1981. n-Alkyl-glucosides as detergents for the preparation of highly homogeneous bilayer liposomes of variable sizes (60-240 nm) applying defined rates of detergent removal by dialysis. Biochem. Biophys. Res. Commun. 100:1055.
29. Papahadjopoulos, D., W. J. Vail, K. Jacobsen, and G. Poste. 1975. Cocleate lipid cylinders: formation by fusion of unilamellar lipid vesicles. Biochim. Biophys. Acta 394:483.
30. Taber, R., T. Wilson, and D. Papahadjopoulos. 1978. The encapsulation of pico-RNA viruses by lipid vesicles: physical and biological properties. Ann. N.Y. Acad. Sci. 308:268.
31. Mannino, R. J., E. S. Allebach, and W. A. Strehl. 1979. Encapsulation of high molecular weight DNA in large, unilamellar phospholipid vesicles. FEBS Lett. 101:229.

32. Papahadjopoulos, D. and W. J. Vail. 1978. Incorporation of macromolecules within large unilamellar vesicles. Ann. N.Y. Acad. Sci. 308:259.
33. Kasahara, M. and P. C. Hinkle. 1977. Reconstitution and purification of the D-glucose transporter from human erythrocytes. J. Biol. Chem. 252: 7384.
34. Pick, U. 1981. Liposomes with a large trapping capacity prepared by freezing and thawing of sonicated phospholipid mixtures. Arch. Biochem. Biophys. (in press).
35. Gebicki, J. M. and M. Hicks. 1973. Ufasomes are stable particles surrounded by unsaturated fatty acid membranes. Nature 243:232.
36. Hargreaves, W. R. and D. W. Deamer. 1978. Liposomes from ionic, single chain amphiphiles. Biochemistry 17:3759.
37. Fry, D. W., C. White, and D. J. Goldman. 1978. Rapid separation of low molecular weight solutes from liposomes without dilution. Anal. Biochem. 90:809.
38. Gunter, K. K., T. E. Gunter, A. Jarkowski, and R. N. Rosier. 1981. A method for resuspending small vesicles separated from suspension by protamine aggregation. Biophys. J. 33:187a.
39. Martin, F. J. and R. C. MacDonald. 1976. Phospholipid exchange between bilayer membrane vesicles. Biochemistry 15:321.
40. Roseman, M. A. and T. E. Thompson. 1980. Mechanism of the spontaneous transfer of phospholipids between bilayers. Biochemistry 19:439.
41. Struck, D. K. and R. E. Pagano. 1980. Insertion of fluorescent phospholipids into the plasma membrane of a mammalian cell. J. Biol. Chem. 255: 5404.
42. Gibson, G. A. and L. M. Loew. 1979. Phospholipid vesicle fusion monitored by fluorescence energy transfer. Biochim. Biophys. Res. Commun. 281:141.
43. Wilschut, J. and D. Papahadjopoulos. 1979. Ca^{82+9-} Induced fusion of phospholipid vesicles monitored by mixing of aqueous contents. Nature 281:690.
44. Uster, P. S. and D. W. Deamer. 1981. Fusion competence of phosphatidylserine-containing liposomes quantitatively measured by a fluorescence resonance energy transfer assay. Arch. Biochem. Biophys. 209:385.

糖鎖工学



産業調査会 バイオテクノロジー情報センター

10. 糖鎖の脂質化工学

水落次男

近年、複合糖質とりわけ糖脂質糖鎖の生理的機能が広く解析されてきているが、その背景として、糖脂質の脂質部分の疎水性を利用した技術の開発が大きく貢献している。なかでも、リポソームの作製技術や、糖脂質をプラスチック表面に不動化したり、薄層クロマトグラフィー（TLC）プレート上で展開分離した後、そこに抗体や細菌やウイルスなどを重層して、それらの糖脂質糖鎖との特異的反応性を調べる技術などの開発を挙げることができる。

一方、糖タンパク質糖鎖の生理的機能の解析は以下に挙げる理由により大きく立ち遅れている。1)生理活性物質がニトロセルロース膜などに不動化した特定の糖タンパク質に特異的に反応しても、タンパク質部分と糖鎖部分のどちらと反応したのかを判定することが困難である。2)生理活性物質がたとえ糖鎖と反応したことが判明しても、糖タンパク質では通常、タンパク質分子上に種々の糖鎖が結合していたり、糖鎖のミクロ不均一性が存在していたりするために、同定した糖タンパク質分子上の果たしてどの糖鎖と反応しているかを特定することがきわめて困難である。3)糖タンパク質より糖鎖をはずし、これを固相に不動化して生理活性物質との反応性を調べようとしても、糖鎖は親水性であるために、そのままでは膜やプレート上に不動化することができない。4)糖タンパク質より得た糖鎖を用いて生理活性物質の反応阻害実験を行うには、生体内に微量にしか存在しない糖タンパク質から構造の均一な糖鎖を大量に単離精製しなければならない。そのため、これまでに示されてきた生理活性物質などの糖タンパク質糖鎖との反応現象の大部分は、主に単糖や化学合成された二糖、三糖を用いた反応阻害実験によって得られた知見であり、実際に生理活性物質などが特異的に反応している糖タンパク質の糖鎖の構造に関する情報はほとんど得られていない。このような事情から、糖タンパク質糖鎖の生理機能を解析するための新しい手法の開発が迫られている。

最近筆者らは、これらの問題点を一挙に解決し、

糖タンパク質糖鎖の機能を解析する新しい手法として「糖タンパク質糖鎖を糖脂質糖鎖として取り扱う」ことを考案し、まだ開発途上の技術ではあるが、糖タンパク質より得たオリゴ糖鎖を脂質（ジパルミトイルホスファチジルエタノールアミンなど）に化学的に結合させ、人工糖脂質を比較的効率よく作製することに成功した[1]。また、この技術を用いて種々の糖タンパク質由来のオリゴ糖鎖を導入した人工糖脂質をオリゴ糖プローブとしてTLCプレート上で展開分離後、プレート上に種々の生理活性物質を重層して人工糖脂質糖鎖との特異的反応性を調べた（図10－1）。その結果、種々の生理活性物質が糖タンパク質の特定の糖鎖構造をその受容体としてそれぞれ識別していることを明らかにできた。また、糖タンパク質糖鎖の生理機能解析におけるこの人工糖脂質の有用性も明らかにできた[1)–5)]。

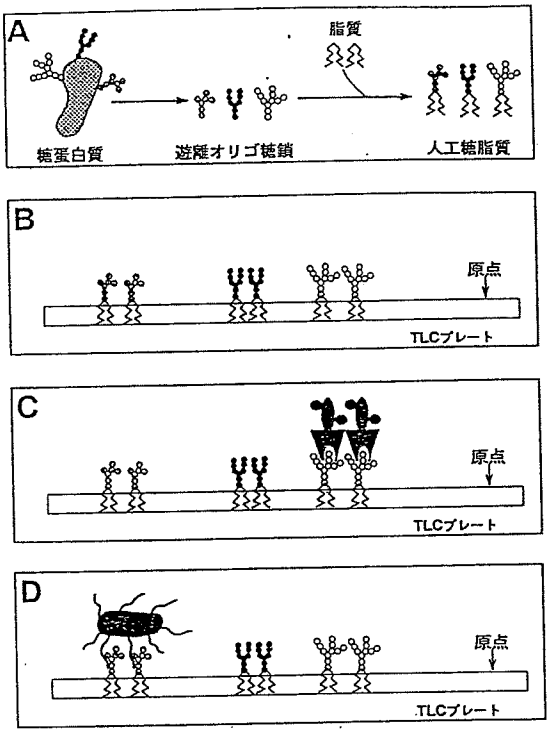


図10－1　糖タンパク質糖鎖の脂質化と人工糖脂質を用いたTLCプレート上での糖タンパク質糖鎖の機能解析
A:糖タンパク質よりオリゴ糖鎖を遊離し、これに脂質を化学的に結合させて人工糖脂質を作製する。B:人工糖脂質はTLCを行なうと、脂質部分は同じであるために人工糖脂質に導入された糖タンパク質糖鎖の構造の違いに基づいて展開分離する。C,D:こうして種々の糖タンパク質糖鎖を含む人工糖脂質が展開分離した、オリゴ糖プローブライブラリーともいえるTLCプレート上にレクチン、モノクローナル抗体、酵素など種々の生理活性物質(C)や、細菌やウイルスなど(D)を重層して反応させた後プレートを洗浄することで、それらの特定の糖タンパク質糖鎖との反応性を調べることができる。

そこで本稿では、糖鎖の脂質化技術とその応用について糖タンパク質糖鎖に焦点を当てて述べる。

10.1　人工糖脂質の特徴

糖タンパク質糖鎖を脂質であるジパルミトイルホスファチジルエタノールアミンなどに化学的に結合させて作製した人工糖脂質は、天然の糖脂質と同様に取り扱うことができる。そのため、糖脂質糖鎖の生理機能解析に利用されている脂質部分の疎水性を利用した技術、すなわち、リポソームの作製技術や、糖脂質をプラスチック表面やTLCプレートなどに不動化し、生理活性物質と反応させる技術などをそっくり糖タンパク質糖鎖の生理機能解析に利用できる。

人工糖脂質では一分子の糖鎖が一分子の脂質に結合している。しかも脂質部分はどの人工糖脂質でも同じであることから、種々の糖鎖を含有している糖タンパク質から遊離して得たオリゴ糖鎖混合物を用いて人工糖脂質を作製後、TLCを行うと、人工糖脂質に導入した糖タンパク質糖鎖の構造の違いに基づいて人工糖脂質を分離することができる（図10－1）。そのため、糖タンパク質から遊離したオリゴ糖鎖を人工糖脂質の作製前にあらかじめ単離精製する必要がなく、人工糖脂質の作製及びその糖鎖機能解析への応用が非常に簡便である。また、糖タンパク質糖鎖を導入した人工糖脂質を糖鎖の構造に基づいてTLCで展開分離した後、プレート上で生理活性物質と直接反応させることにより、糖タンパク質糖鎖のうちのどの糖鎖が特異的に反応したかを明らかにできる。そのため、この人工糖脂質は糖タンパク質糖鎖の生理機能解析のためのオリゴ糖プローブとして非常に有用である。

10.2　糖鎖の脂質化

糖タンパク質糖鎖を脂質に化学的に結合させて人工糖脂質を作製する試みはこれまでにもいくつか行われてきたが、それらは、産物である人工糖脂質を免疫学的には同定できても化学的にはほとんど検出

できないほど反応効率が悪いものであった。筆者らは、ヒドラジン分解法により種々の糖タンパク質から遊離したオリゴ糖鎖をジパルミトイルホスファチジルエタノールアミンという脂質に還元アミノ化反応を用いて結合させて新しい人工糖脂質を比較的効率よく作製することに成功し、糖タンパク質糖鎖を糖脂質糖鎖として取り扱うことを可能にした[1]。以下に筆者らが通常行っている糖タンパク質糖鎖の人工糖脂質への導入法とその分離・検出などについて述べる。

（1）人工糖脂質の作製法

人工糖脂質の作製には、ヒドラジン分解法などの化学的手法やグリコペプチダーゼなどを用いた酵素学的手法によって種々の糖タンパク質や糖ペプチドより遊離して得た還元末端アルデヒド基を含むオリゴ糖鎖や、尿や乳汁などより調製した遊離オリゴ糖鎖を用いる。この人工糖脂質作製反応では、オリゴ糖鎖の還元末端アルデヒド基が脂質であるジパルミトイルホスファチジルエタノールアミンのアミノ基との間にシッフ塩基を作り、これが$NaBH_3CN$で還元されて人工糖脂質が生成する。

以下にその作製例を述べる。まず、種々のオリゴ糖鎖（25～50μg）に6μlの蒸留水を加えて撹拌溶解する。これに、5 mg/ml濃度でクロロホルム－メタノール（1：1,v/v）に溶解したジパルミトイルホスファチジルエタノールアミン溶液を94μlと10mg/ml濃度でメタノールに溶解した$NaBH_3CN$溶液を10μl加えて撹拌混合する。次に、この反応混合液を60℃で16時間保温して、オリゴ糖鎖を導入した人工糖脂質を作製する。

この方法を用いると、ラクトースやシアリルラクトースはほぼ100%脂質に結合する。また、糖タンパク質由来の中性オリゴ糖鎖は60～70%が脂質に結合するが、糖タンパク質由来でシアル酸などを含む酸性オリゴ糖鎖では脂質への結合量がそれよりも低下することが判明している。そこで現在筆者の研究室では、糖タンパク質由来酸性オリゴ糖鎖を人工糖脂質へ効率よく導入する反応条件を種々検討しており、より良い反応条件を得つつある。

（2）薄層クロマトグラフィー（TLC）による人工糖脂質の分離とその検出

人工糖脂質は天然の糖脂質と同様に取り扱うことができるので、天然の糖脂質の分離によく使用されるクロロホルム－メタノール－水系の展開溶媒を用いたTLCで、種々の糖タンパク質糖鎖を含む人工糖脂質混合物を糖鎖構造の違いに基づいて分離できる。また、TLCプレート上の人工糖脂質糖鎖は化学的手法、生化学的手法、免疫化学的手法、生物学的手法などを用いて検出できるため、対象とする糖タンパク質の糖鎖機能解析をこのTLCプレート上で行うことができる。

筆者らは通常、人工糖脂質作製反応混合物をTLCで分離するために、Merck社のHPTLCプレートを使用し、展開溶媒には人工糖脂質に導入したオリゴ糖鎖のサイズに応じて、クロロホルム－メタノール－水の混合比が60：35：8 (v/v)、55：45：10(v/v)、105：100：28 (v/v)、50：55：18 (v/v) のものを適当に使い分けている。なお、TLCプレート上に展開分離した人工糖脂質の化学的検出には天然糖脂質の分析に通常使用されている脂質染色法や糖質染色法を用いている。図10－2に種々のオリゴ糖鎖及び、その人工糖脂質作製反応混合物のTLCによる分離とその化学的検出の実例を示してある。

また、抗体、レクチン、細菌、ウイルス、種々の

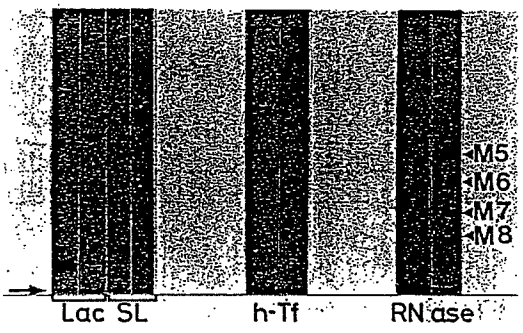


図10－2　人工糖脂質のTLCによる分離と人工糖脂質の糖染色

ラクトース(Lac)、シアリルラクトース(SL)、シアル酸を結合した二本鎖complex型糖鎖を含有するヒトトランスフェリン糖鎖(h-Tf)、一連のhigh mannose型糖鎖(図10－3D)を含有するウシリボヌクレアーゼ糖鎖(RNase)をそれぞれ脂質化後、TLCにて展開分離し、TLCプレートをオルシノール硫酸にて糖染色した。各左側のレーンがオリゴ糖鎖のみを展開分離したもの。各右側のレーンが人工糖脂質作製反応混合物を展開分離したもの。展開溶媒にはクロロホルム/メタノール/水を使用し、その混合比は、LacとSLは60:35:8(v/v)を、h-Tfは50:55:18(v/v)を、RNaseは105:100:28(v/v)を用いた。

生理活性物質などをTLCプレート上に重層することでそれらが反応する人工糖脂質糖鎖を検出できるし、さらに、種々のグリコシダーゼ溶液をTLCプレート上に重層して保温することで、人工糖脂質糖鎖の*in situ*でのグリコシダーゼ処理ができる。これらの実験例については後で紹介する。

(3) 人工糖脂質の構造分析

人工糖脂質は天然の糖脂質と同様に取り扱うことができるので、天然糖脂質の構造解析に用いられている種々の手法を使用して人工糖脂質の構造解析を行うことができる。とりわけ、TLCプレート上に展開分離した人工糖脂質をSI-MS法やFAB法を用いた質量分析計で直接分析する方法が簡便であり、しかも非常に効果的である。糖タンパク質由来の遊離オリゴ糖鎖を導入した人工糖脂質は遊離オリゴ糖鎖と比較してイオン化しやすい。そのため、反応混合物をTLCで展開分離後、目的の人工糖脂質のバンド部分をTLCプレートから切り出してFAB-MSあるいはSI-MSのターゲットに直接はりつけ、マトリックスを付加して、イオン化し、質量分析することでTLCプレート上の特定の人工糖脂質の構造を容易に調べることができる[1)6)]。また、TLCプレート上での人工糖脂質の*in situ*グリコシダーゼ消化とともに、その質量変化や、糖鎖特異抗体やレクチンなどに対する反応性の変化を調べて人工糖脂質糖鎖の構造を解析することもできる。

10.3 糖タンパク質糖鎖機能解析への人工糖脂質の応用

筆者らはこれまでに、種々の糖タンパク質由来オリゴ糖鎖を導入した人工糖脂質をオリゴ糖プローブとして用いてTLCプレート上で展開分離後、プレート上に植物レクチン、ウシコングルチニン、ヒト血清由来マンノース結合タンパク質、尿路感染症患者由来病原性大腸菌などを重層して、人工糖脂質糖鎖との特異的反応性を調べた(図10－1)[1)-5)]。その結果、生理活性物質や病原菌などが糖タンパク質の特定の糖鎖構造をそれぞれ識別していることを明らかにし、糖タンパク質糖鎖の生理機能解析におけるこの人工糖脂質の有用性を明らかにすることができた。そこで本節では、糖タンパク質糖鎖の生理機能解析への人工糖脂質の応用例を紹介する。

(1) ウシコングルチニンによる糖タンパク質糖鎖の特異的識別

ウシコングルチニンはCa^{2+}存在下でザイモサン(Zymosan)や補体成分であるiC3bに結合する血清中のレクチンである。筆者らはどのような糖鎖がこのレクチンの受容体として機能しているかを、種々の糖タンパク質由来糖鎖を導入した人工糖脂質をオリゴ糖プローブとして用いて調べた[1)]。なお、図10－1(A－C)にその概略を漫画風に示してある。

まず、complex型糖鎖を含有するヒトIgG[7)]、マウスIgG[8)]、ヒトトランスフェリン[9)]、high mannose型糖鎖を含有するウシリボヌクレアーゼB[10)]、high mannose型糖鎖とhybrid型糖鎖の両方を含有する卵白アルブミン[11)]をそれぞれヒドラジン分解にかけて、*N*-グリコシド型糖鎖をタンパク質部分より遊離させてオリゴ糖鎖を精製した。ヒトIgG、マウス

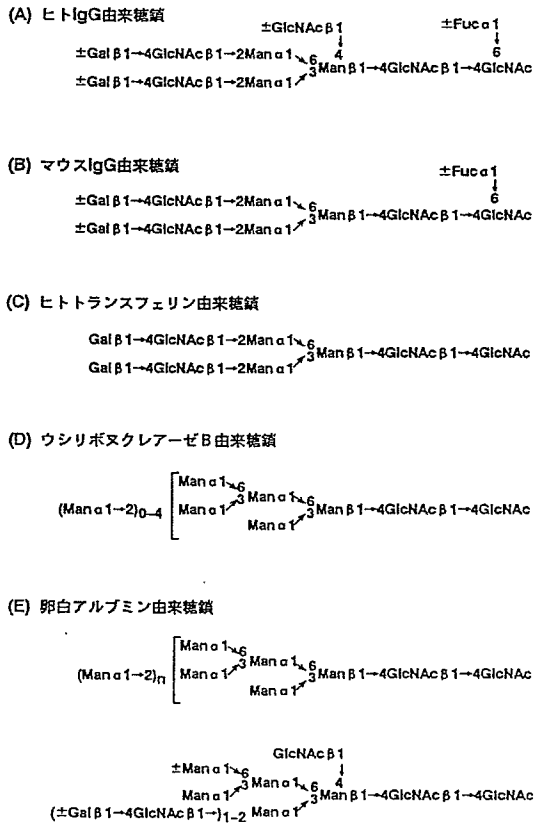


図10－3 糖タンパク質由来オリゴ糖鎖の構造
図中のGalはガラクトースを、GlcNAcは*N*-アセチルグルコサミンを、Manはマンノースを、Fucはフコースをそれぞれ示す。

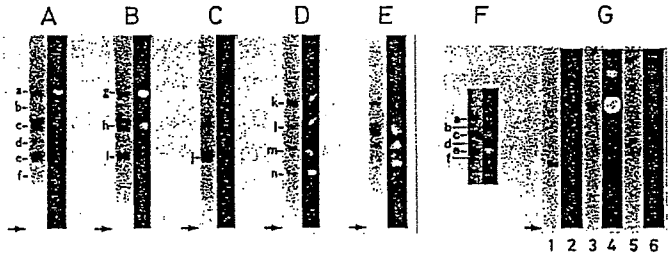


図10－4　ウシコングルチニンによる糖タンパク質糖鎖の特異的識別

IgG、ヒトトランスフェリンの糖鎖はさらにシアリダーゼ消化して脱シアル化オリゴ糖鎖とした。次に、筆者らが開発した脂質化技術を使って、それらの糖鎖（図10－3にその構造を示してある）を導入した人工糖脂質を作製し、さらにこれをTLCにて展開分離した。種々の糖鎖を含有する人工糖脂質を展開分離して作製した、オリゴ糖プローブライブラリーとも言えるこのプレート上に^{125}I-標識したコングルチニンを重層して一定時間反応後、プレートを洗浄・乾燥し、オートラジオグラフィを行って、コングルチニンの受容体となる人工糖脂質の位置を同定した（図10－4のパネルA～Fの右側のレーン）。この後、同一プレートをオルシノール硫酸にて糖染色してプレート上のすべての人工糖脂質の位置を同定した（図10－4のパネルA～Fの左側のレーン）。なお、図10－4のパネルA～Eは図10－3のA～Eに示す糖鎖をそれぞれ導入した人工糖脂質を展開分離したものである。また、図10－4にa～nで示した人工糖脂質バンドの糖鎖構造は、前述のように、TLCプレートの各バンド部分を切出し、直接SI-MS分析することでそれぞれ同定した。

図10－4のA～Eをみると、ウシコングルチニンは、iC3bにも存在する糖タンパク質のhigh mannose型糖鎖（図10－4D,バンドk～n）の他にも、hybrid型糖鎖（図10－4E）やcomplex型の特定の糖鎖（図10－4A～C,バンドa,c,g,h）にもよく結合することがわかる。また、糖染色の結果とオートラジオグラフィの結果の比較から、ウシコングルチニンは、1) high mannose型糖鎖ではManα1→2残基を多く含む糖鎖（$Man_{8,7}GlcNAc_2$,バンドn,m）により強く結合すること、2) complex型糖鎖では非還元末端*N*-アセチルグルコサミン（GlcNAc）残基を持たない糖鎖（バンドe,i,j）には結合しないが、1個含む糖鎖（バンドc,h）と2個含む糖鎖（バンドa,g）には結合し、とりわけ非還元末端GlcNAcを2残基含む糖鎖（バンドa,g）に強く結合すること、しかし、3) そのような糖鎖でもbisecting GlcNAc残基を含むと（バンドb,d）結合しなくなることなどがわかる。

図10－4Fは図10－4Aと同じく図10－3Aに示した糖鎖を導入した人工糖脂質を展開分離してあるが、まず、プレート上にβ-ガラクトシダーゼを重層して、プレート上の人工糖脂質を*in situ*で酵素処理し、次にプレートを洗浄し、^{125}I-標識したコングルチニンを重層して反応させた結果を示している。これを見ると、図10－4Aではコングルチニンの受容体になり得なかった非還元末端Galを2残基含む糖鎖（図10－3A）を持つ人工糖脂質バンドeが*in situ* β-ガラクトシダーゼ処理によりガラクトースを遊離し、GlcNAcを非還元末端に露出した結果、コングルチニンの受容体になったことがわかる。

図10－4Gでは、図10－3Cに示すオリゴ糖鎖（レーン1,2）、それをβ-ガラクトシダーゼ処理してガラクトースを除去したオリゴ糖鎖（レーン3,4）、さらにβ-*N*-アセチルグルコサミニダーゼ処理してGlcNAcも除去したオリゴ糖鎖（レーン5,6）をそれぞれ導入した人工糖脂質が展開分離してある。これを見ると、コングルチニンはガラクトースが2残基ともGlcNAcに結合して、GlcNAcを非還元末端側に露出していないオリゴ糖鎖（図10－3C）を導入した人工糖脂質にはまったく結合しないが（レーン2）、この糖鎖がβ-ガラクトシダーゼ処理によりガラクトースを遊離してGlcNAcを非還元末端側に露出したオリゴ糖鎖になると、コングルチニンが非常に強く結合するようになることがわかる（レーン4）。また、さらにβ-*N*-アセチルグルコサミニダーゼ処理によりそのGlcNAc残基を除去して$Man_3GlcNAc_2$という糖鎖にしてしまうとコングルチニンの結合能が極端に低下することがわかる（レーン6）。

結局、1) ウシコングルチニンの受容体として糖タンパク質のhigh mannose型糖鎖以外にもhybrid型

糖鎖やcomplex型の特定の糖鎖が機能していること、2) high mannose型糖鎖ではManα1→2残基含有糖鎖がより強い受容体活性を示すこと、3)hybrid型やcomplex型の糖鎖が受容体として機能するためには非還元末端のGlcNAcやマンノースの存在が必須であるが、とりわけ非還元末端GlcNAcを含む糖鎖は非常に強い受容体活性を示すこと、しかし、4) bisecting GlcNAcを含む糖鎖は受容体にはならないこと、などが糖タンパク質糖鎖を導入した人工糖脂質をオリゴ糖プローブとして用いたこの研究で、初めて明らかにされた。また、人工糖脂質のTLCプレート上での*in situ*グリコシダーゼ消化が可能であることも示された。

(2) 血清中に存在するマンノース結合タンパク質の受容体として機能するエイズウイルス表面糖タンパク質糖鎖の同定

エイズの原因ウイルスであるHIVの表面は、約50%の糖含量を示すウイルスエンベロープの糖タンパク質であるgp120分子によって覆われている。HIVによる感染はこの分子が宿主細胞表面のCD4分子に結合することで生じる。糖鎖分解酵素を用いた研究や、糖タンパク質の*N*-グリコシド型糖鎖の生合成やプロセッシングの阻害剤を用いた研究などにより、HIV感染過程におけるウイルス表面糖タンパク質糖鎖の関与が示されてきている[12]。筆者らは、HIV感染過程に関与する糖鎖を明らかにする目的で、HIV表面の主要な糖タンパク質であるgp120の糖鎖構造を解析し、HIV-1 (IIIB) 感染H9細胞由来のgp120の脱シアル化糖鎖が図10－5に示す多様な糖鎖構造を有していることを明らかにした[13]。また、これらの糖鎖のうちでHIV感染に重要と思われる糖鎖も同定することができた[12]。

図10－5に示したように、HIV感染細胞由来gp120の糖鎖の6割が$Man_{9-5}GlcNAc_2$という一連のhigh mannose型糖鎖であるという事実は、gp120が

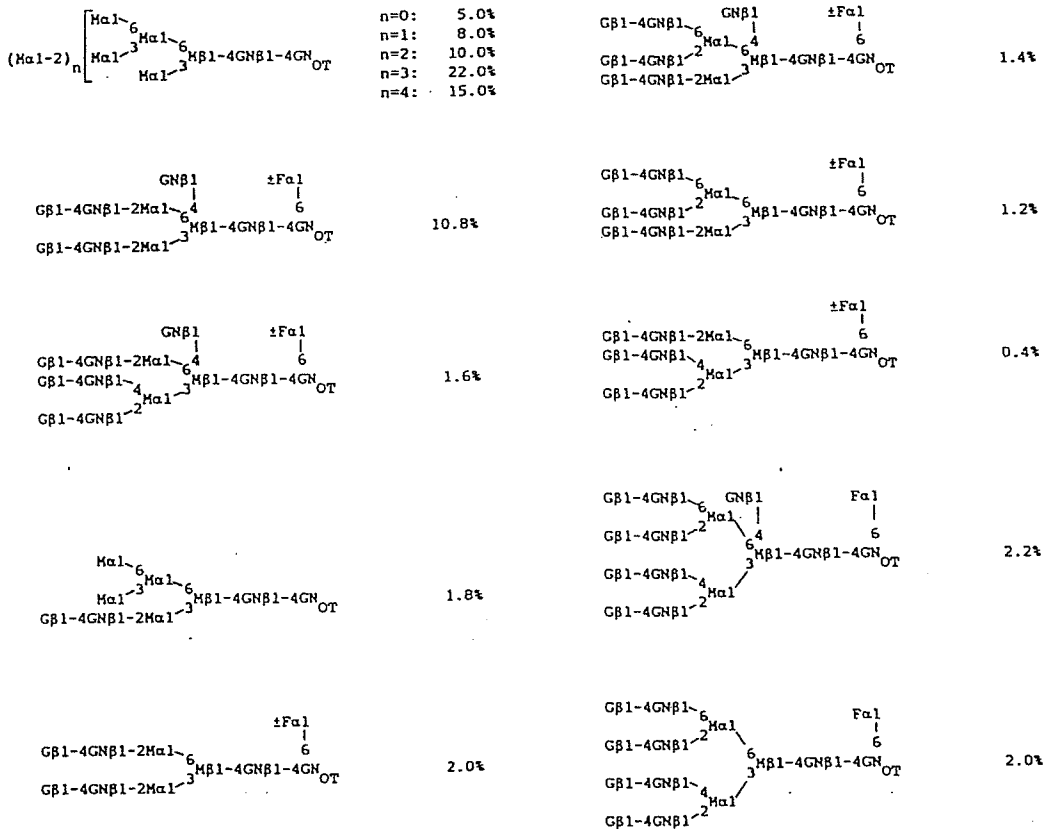


図10－5　HIV-1(IIIB)感染H9細胞由来gp120の糖鎖構造
図中のGはガラクトースを、GNは*N*-アセチルグルコサミンを、Mはマンノースを、Fはフコースをそれぞれ示す。

ウイルス表面を覆っている糖タンパク質であり、*N*-グリコシド型糖鎖の結合部位を24カ所も有し、しかもgp120分子の約半分が糖鎖であることを考え合わせると、HIV表面にはこのhigh mannose型糖鎖がかなり露出していることを意味している。また、ヒト血清中にはマンノースに親和性を示すマンノース結合タンパク質というCa^{2+}依存性のレクチンの存在が知られており、しかもこのレクチンは受容体に結合すると補体系を活性化することも知られている[14)15)]。そのため、gp120がこのレクチンに糖鎖を介して結合することが十分考えられたので、実際に、ヒト血清マンノース結合タンパク質がgp120に結合するかを調べてみたところ、確かに結合することが示された[3)]。

そこで次に筆者らは、gp120糖鎖のうちでどの糖鎖がヒト血清中のマンノース結合タンパク質の受容体として機能しているかを明らかにするために、gp120のオリゴ糖鎖を導入した人工糖脂質をオリゴ糖プローブとして用いた研究を実施した（図10－6）[3)]。なお、この実験の概略は図10－1（A～C）に漫画風に示してある。まず、HIV-1(IIIB)感染H9細胞のgp120及びチャイニーズハムスター卵巣（CHO）細胞で遺伝子工学的に産生させたHIV-1（IIIB）のリコンビナントgp120（rgp120）[16)17)]をヒドラジン分解にかけて*N*-グリコシド型糖鎖を遊離して精製した。この2種類のgp120糖鎖と、図10－3Dに示す一連のhigh mannose型糖鎖を有するウシリボヌクレアーゼ糖鎖(RNase)、complex型糖鎖を有するヒトIgG由来糖鎖（図10－3A）を脂質化技術を用いて人工糖脂質に導入した。次にこれらの人工糖脂質を展開分離したTLCプレート上に^{125}I-標識したコンカナバリンA（ConA）またはヒト血清マンノース結合タンパク質（sMBP）を重層して反応させ、オートラジオグラフィを行って、ConAやsMBPの受容体となる糖鎖を含有する人工糖脂質の位置を同定した。この後、同一プレートをオルシノール硫酸により糖染色してすべての人工糖脂質の位置を同定した（orcinol）。

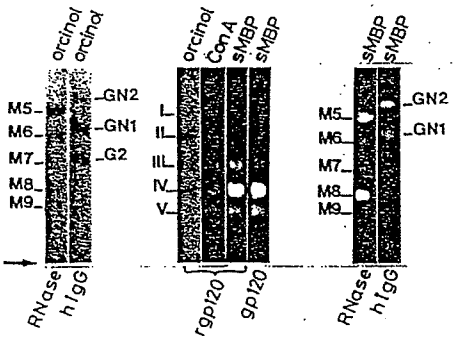


図10－6　ヒト血清マンノース結合タンパク質の受容体として機能するエイズウイルス表面糖タンパク質gp120の糖鎖

図10－6にその結果を示してあるが、マンノースに親和性を示すConAはrgp120に含まれる一連のhigh mannose型糖鎖[16)17)]を受容体とした。しかし、マンノース結合タンパク質の場合はそれとは異なって、rgp120及び感染細胞由来gp120に含まれる一連のhigh mannose型糖鎖のうち$Man_{9-7}GlcNAc_2$を受容体としており、しかも$Man_8GlcNAc_2$が非常に強い受容体活性を有していることが明らかにされた。このことは、同時に調べた一連のhigh mannnose型糖鎖（図10－3D）を導入した人工糖脂質（RNase）で、糖染色の強度と受容体活性の強度を比較してみても明らかである。

以上の知見と、マンノース結合タンパク質がその受容体に結合すると抗体を介さずに補体系を活性化できるという報告[14)15)]を考え合わせると、HIV感染に際してHIVあるいはHIV感染細胞がヒトの血液中に入った時点で、血液中に存在するマンノース結合タンパク質が、その受容体であるHIVあるいは感染細胞表面を覆っているgp120の糖鎖のうちで$Man_{9-7}GlcNAc_2$とりわけ$Man_8GlcNAc_2$という糖鎖に非常に強く結合し、抗体を介さずに補体系を活性化し、HIV感染の初期防御に関係しているとも考えられる。しかし、実際には感染が進行してしまうので、HIV粒子や感染細胞の表面にはHRF-20などの補体反応系を阻害する物質が存在する可能性や、この現象が補体系を介した感染の促進に関与している可能性も考えられる。

HIV表面糖タンパク質gp120の糖鎖を導入した人工糖脂質をオリゴ糖プローブとして用いて初めて得られたこれらの知見は、HIV感染やエイズの発症を阻止する方法を考える上で非常に重要である。今後、血清中のマンノース結合タンパク質がHIV感染の初期防御に関与しているのか感染の促進に関与しているのかを明らかにする必要があろう。

（3）尿路感染症患者の病原性大腸菌が付着に際して識別する糖タンパク質の糖鎖

病原菌による感染に際して、病原菌はまず宿主に

付着・定着し、そして侵入すると考えられている。そこで筆者らは尿路感染症患者由来の病原性大腸菌が宿主細胞表面に付着する過程を糖タンパク質糖鎖に着目して解析した。種々の糖タンパク質糖鎖を導入した人工糖脂質をオリゴ糖プローブとして用いて解析を行った結果、この大腸菌が宿主に付着する過程で糖タンパク質の特定の糖鎖を受容体としていることを明らかにすることができた[4)5)]。

筆者らは、細胞表面糖タンパク質糖鎖と同様の糖鎖を含むと考えられる種々の糖タンパク質からヒドラジン分解法により得たオリゴ糖鎖を、脂質化技術を用いて人工糖脂質に導入した。そして、これら種々の人工糖脂質を展開分離して作製した、オリゴ糖プローブライブラリーとも言えるTLCプレート上に^{14}C-グルコースで標識した病原性大腸菌を重層して一定時間静置後、プレートを洗浄・乾燥し、フルオログラフィーを行って、この大腸菌が付着した人工糖脂質の位置を検出した（図10－7中のFで示したレーン）。また、同一プレートをオルシノール硫酸にて糖染色した（図10－7中のOで示したレーン）。図10－1（A,B,D）にこの手法の概略を漫画風に示してある。なお図10－7では、マンノースによって血球凝集阻害を受ける尿路感染症患者由来の病原性大腸菌C600株を使用している[4)]。

図10－3のA～Dで示す糖鎖を含有する人工糖脂質をオリゴ糖プローブとして用いて調べた結果（図10－7,a～d）、この病原性大腸菌は一連のhigh mannose型糖鎖（図10－3D）をその受容体として識別して付着し、とりわけ$Man_5GlcNAc_2$という糖鎖（M5）に非常に強く付着したが（図10－7d）、それ以外の図10－3のA～Cに示すcomplex型糖鎖は受容体にはならないことが示された（図10－7,a～c）。図10－3Dの糖鎖を含む人工糖脂質をレーンdの二倍量展開分離しても同様の結果が得られるが（図10－7f）、10mMマンノース存在下ではこの大腸菌による糖鎖受容体識別現象が阻害されることも示された（図10－7g）。一方、この大腸菌の受容体にはならなかった図10－3Cの糖鎖も、ガラクトースと*N*-アセチルグルコサミンを除去して$Man_3GlcNAc_2$というマンノースを非還元末端に持つ糖鎖にすると非常に強い受容体活性を示すようになることも明らかとなった（図10－7c,e）。

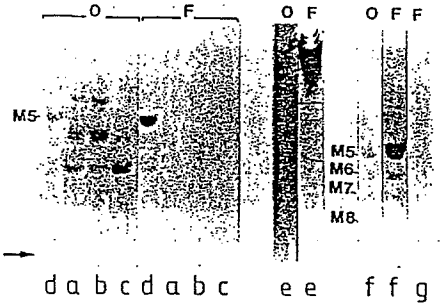


図10－7　尿路感染症患者の病原性大腸菌が付着に際して識別する糖タンパク質の糖鎖

種々の糖タンパク質由来のオリゴ糖鎖を導入した人工糖脂質を展開分離してあるオリゴ糖プローブライブラリーとも言えるTLCプレートを用いて初めて、この病原性大腸菌C600株が、非還元末端マンノースを含むhigh mannnose型糖鎖のうちとりわけ$Man_5GlcNAc_2$を受容体として識別しており、マンノースが糖鎖の内側にあるcomplex型糖鎖には受容体活性がないことが明らかにされたわけである。また最近筆者らは、種々の尿路感染症患者から分離したマンノースによって血球凝集阻害を受ける大腸菌株を多数集め、“オリゴ糖プローブライブラリー”を用いて糖タンパク質糖鎖の受容体活性を調べている。その結果、ある種の大腸菌株に対してはC600株の受容体とは異なる糖鎖にも受容体活性があることを示唆する知見を得ている[5)]。

本稿では糖鎖の脂質化技術、とりわけ糖タンパク質糖鎖を導入した人工糖脂質の作製とその性質、それをオリゴ糖プローブとして用いたTLCプレート上での糖タンパク質糖鎖の生理機能解析例について述べた。本稿で紹介した糖鎖の脂質化技術を用いて作製した人工糖脂質はTLCプレート上で利用できるだけでなく、プラスチック表面への不動化やリポソームの作製も可能である。また今後、脂質部分の疎水性を利用した他の技術もさらに開発されるであろう。そのため、この人工糖脂質はレクチンによる特異的糖鎖識別現象の解析だけではなく、モノクローナル抗体や糖転移酵素、細胞接着因子など様々な生理活性物質による精密な糖鎖構造識別現象の解析、糖タンパク質糖鎖に対するモノクローナル抗体の作製、細胞間識別現象における細胞表面糖タンパク質糖鎖の生理機能解析などにもオリゴ糖プローブとして使用することができる。糖タンパク質糖鎖を導入した人工糖脂質は、単糖鎖や二糖類を用いた阻害実験などでは解明できなかった、より精密な糖鎖構造

識別現象を明らかにするためのオリゴ糖プローブとしてその意義は非常に大きいといえる。また、この人工糖脂質は糖鎖に着目した医薬品の開発などにも今後大きく貢献するものと思われる。

〔参考文献〕

1) Mizuochi,T.,Loveless,R.W.,Lawson,A.M.,Chai,W., Lachmann,P.J.,Childs,R.A.,Thiel,S.,and Feizi,T. (1989) J.Biol.Chem.264,13834-13839
2) Childs,R.A.,Drickamer,K.,Kawasaki,T.,Thiel,S., Mizuochi,T.,and Feizi,T. (1989) Biochem.J.262, 131-138
3) Larkin,M.,Childs,R.A.,Matthews,T.J.,Thiel,S., Mizuochi,T.,Lawson,A.M.,Savill,J.S.,Haslett,L., Diaz,R.,and Feizi, T. (1989) AIDS,3,793-798
4) Rosenstein,I.J.,Stoll,M.S.,Mizuochi,T.,Childs,R.A., Hounsell,E.F.,and Feizi,T. (1988) The Lancet,ii,1327-1330
5) 水落次男・加藤真理・川合可子・浜子二治・千谷晃一・友近健一・小林直樹・金政泰弘・保田立二 (1990) Bacterial Adherence研究、4,36-43
6) Kushi,Y.and Handa,S. (1985) J.Biochem.98,265-268
7) Mizuochi,T.,Taniguchi,T.,Shimizu,A,and Kobata, A. (1982) J.Immunol.129,2016-2020
8) Mizuochi,T.,Hamako,J.,and Titani,K. (1987) Arch. Biochem.Biophys.257,387-394
9) Spik,G.,Bayard,B.,Fournet,B.,Strecker,G., Bouquelet,S.,and Montreuil,J. (1975) FEBS Lett.50, 296-299
10) Liang,C.-J.,Yamashita,K.,and Kobata,A. (1980) J. Biochem.88,51-58
11) 木幡陽 (1979) タンパク質核酸酵素、24,119-130
12) 水落次男 (1992) 日本臨床、50、印刷中
13) Mizuochi,T.,Matthews,T.J.,Kato,M.,Hamako,J., Titani,K.,Solomon,J.,and Feizi,T. (1990) J.Biol. Chem.265,8519-8524
14) Kuhlman,M.,Joiner,K.,and Ezekowitz,R.A.B. (1989) J.Exp.Med.169,1733-1745
15) Ohta,M.,Okada,M.,Yamashina,I.,and Kawasaki,T. (1990) J.Biol.Chem.265,1980-1984
16) Mizuochi,T.,Spellman,M.W.,Larkin,M.,Solomon, J.,Basa,L.J.,and Feizi,T. (1988) Biochem.J.254,599-603
17) Mizuochi,T.,Spellman,M.W.,Larkin,M.,Solomon, J.,Basa,L.J.,and Feizi,T. (1988) Biomed.Chromatogr. 2,260-270